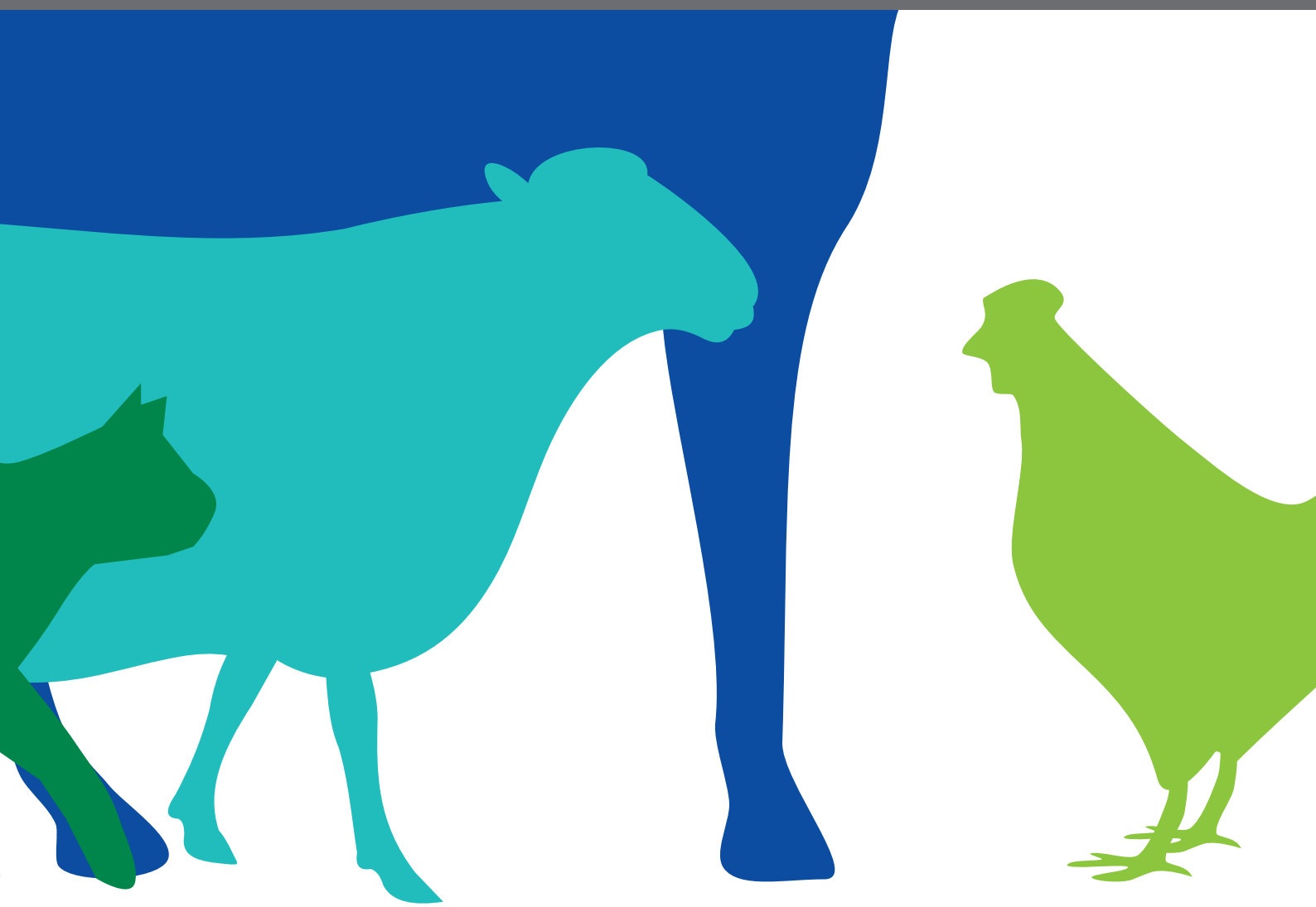
BUFFALO HEALTH AND PRODUCTION

EDITED BY: Antonio Minervino, Selwyn Arlington Headley, André Mendes Jorge and Domenico Vecchio

PUBLISHED IN: Frontiers in Veterinary Science



frontiers Research Topics

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ISSN 1664-8714
ISBN 978-2-88974-869-3
DOI 10.3389/978-2-88974-869-3

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BUFFALO HEALTH AND PRODUCTION

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Citation: Minervino, A., Headley, S. A., Jorge, A. M., Vecchio, D., eds. (2022). Buffalo Health and Production. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88974-869-3

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frontiers in Veterinary Science

EDITORIAL
published: 11 February 2022
doi: 10.3389/fvets.2021.810923

Editorial: Buffalo Health and Production

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Keywords: buffalo, animal health, animal reproduction, animal science, *Bubalus bubalis*, animal infectious diseases

Editorial on the Research Topic

Buffalo Health and Production

The domestic buffalo (*Bubalus bubalis*), also known as water buffalo or Asian buffalo comprises two subspecies: the river buffalo (*B. bubalis bubalis*) and the swamp buffalo (*B. bubalis kerebau*) (1). Considering buffalo characteristics such as rusticity and productivity, we believe that buffalo production should be expanded worldwide, especially in developing countries with adequate natural conditions (2). This amazing and underused animal can become more productive and should be promoted as a target species to be used in smallholder and sustainable production systems (3).

To provide a venue for quality research on buffalo this Research Topic on ***Buffalo Health and Production*** has a deliberately broad scope to allow a wide range of articles to be published. A total of 29 papers are included, with three review articles, 21 original research articles, and five brief research reports from 223 authors from around the globe. **Figure 1** presents a word cloud with the most common words used in the manuscript titles.

Two papers comprise comprehensive reviews of important buffalo diseases. The first review from de Barros et al. evaluates the available published literature of *Toxoplasma gondii* and *Neospora caninum* in buffalos and related serological evidence on these parasitic diseases in buffalos from most continents. The second review of parasite infections from de Aquino et al. presents an overview of the occurrence of cryptosporidiosis and giardiasis in water buffaloes and demonstrates that *Cryptosporidiu*m spp. and assemblages of *Giardia duodenalis* and may be sources for potential zoonotic infections. Moreover, the review of published data suggests that young buffalo are more prone to infection than their older counterparts and that infected buffalos can be asymptomatic or with clinical manifestations of these protozoan diseases.

An additional review paper from Minervino et al. presented the basic aspects of the water buffalo and unraveled the buffalo path followed from the origin of the species to its current global distribution, providing a more accurate estimate of the world buffalo count and distribution.

Considering original/brief research, the Research Topic published five articles related to infectious disease, one describing infection with *Leptospira* spp. in Brazil from Guedes et al. and an interesting study from Esposito et al. showing abortions due to *Listeria monocytogenes* in Italy, indicating that *Listeria* must be included as a possible cause of abortions in buffalo herds. The remaining infectious disease articles address new diagnostic tools to detect infections, such as the use of an interferon-gamma assay as a diagnostic strategy for *Mycobacterium bovis* in water buffalos from Martucciello et al.; a pulsed-field gel electrophoresis to detect Salmonella described by Santana et al.; and a flow cytometry to Study Leukocyte alterations during BVDV Acute Infection proposed by Grandoni et al..

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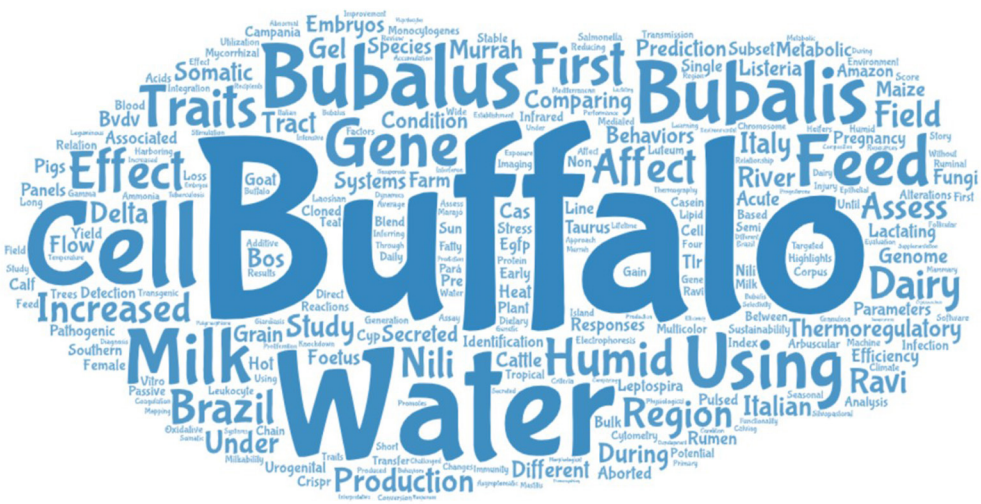
Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *08 November 2021*
Accepted: *07 December 2021*
Published: *11 February 2022*

Citation:
Minervino AHH, Vecchio D, Jorge AM and Headley SA (2022) Editorial: Buffalo Health and Production. Front. Vet. Sci. 8:810923. doi: 10.3389/fvets.2021.810923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ORIGINAL RESEARCH
published: 23 April 2020
doi: 10.3389/fvets.2020.00199

Generation of Transgenic Cloned Buffalo Embryos Harboring the EGFP Gene in the Y Chromosome Using CRISPR/Cas9-Mediated Targeted Integration

Xiuling Zhao[1†], Junyu Nie[1†], Yuyan Tang[1], Wengtan He[1], Kai Xiao[1], Chunying Pang[2], Xianwei Liang[2], Yangqing Lu[1] and Ming Zhang[1*]*

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OPEN ACCESS

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Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: 18 February 2020
Accepted: 25 March 2020
Published: 23 April 2020

Citation:
Zhao X, Nie J, Tang Y, He W, Xiao K, Pang C, Liang X, Lu Y and Zhang M (2020) Generation of Transgenic Cloned Buffalo Embryos Harboring the EGFP Gene in the Y Chromosome Using CRISPR/Cas9-Mediated Targeted Integration. Front. Vet. Sci. 7:199. doi: 10.3389/fvets.2020.00199

Sex control technology is of great significance in the production of domestic animals, especially for rapidly breeding water buffalo (bubalus bubalis), which served as a research model in the present study. We have confirmed that a fluorescence protein integrated into the Y chromosome is fit for sexing pre-implantation embryos in the mouse. Firstly, we optimized the efficiency of targeted integration of exogenous gene encoding enhanced green fluorescent protein (eGFP) and mCherry in Neuro-2a cells, mouse embryonic stem cells, mouse embryonic cells (NIH3T3), buffalo fetal fibroblast (BFF) cells. The results showed that a homology arm length of 800 bp on both sides of the target is more efficient that 300 bp or 300 bp/800 bp. Homology-directed repair (HDR)-mediated knock-in in BFF cells was also significantly improved when cells were supplemented with pifithrin-μ, which is a small molecule that inhibits the binding of p53 to mitochondria. Three pulses at 250 V resulted in the most efficient electroporation in BFF cells and 1.5 μg/mL puromycin was found to be the optimal concentration for screening. Moreover, Y-Chr-eGFP transgenic BFF cells and cloned buffalo embryos were successfully generated using CRISPR/Cas9-mediated gene editing combined with the somatic cell nuclear transfer (SCNT) technique. At passage numbers 6–8, the growth rate and cell proliferation rate were significantly lower in Y-Chr-eGFP transgenic than in non-transgenic BFF cells; the expression levels of the methylation-related genes *DNMT1* and *DNMT3a* were similar; however, the expression levels of the acetylation-related genes *HDAC1*, *HDAC2*, and *HDAC3* were significantly higher ($p < 0.05$) in Y-Chr-eGFP transgenic BFF cells compared with non-transgenic cells. Y-Chr-eGFP transgenic BFFs were used as donors for SCNT, the results showed that eGFP reporter is suitable for the visualization of the sex of embryos. The blastocyst rates of cloned buffalo embryos were similar; however, the cleavage rates of transgenic cloned embryos were significantly lower compared with control. In summary, we optimized the protocol for generating transgenic BFF cells and successfully generated Y-Chr-eGFP transgenic embryos using these cells as donors.

Keywords: sex control, CRISPR/Cas9, HDR, Y chromosome, p53, PFT-μ

INTRODUCTION

Producing offspring with the desired sex is a significant goal in livestock production. Several approaches have been used to determine the sex of embryos prior to embryo transfer in mammals. Histocompatibility-Y antigen, which serves as a male-specific antigen, has been used to identify the gender of bovine embryos (1). Non-radioactive *in situ* hybridization (2) and fluorescence *in situ* hybridization (3) using a labeled Y-specific probe have been performed to determine the sex of human and bovine embryos. Sex chromosome-based PCR analysis has also been used to determine the sex of embryos (4–6). However, these methods are generally error prone, labor intensive and can even be detrimental to normal embryonic development (7, 8). Therefore, it is of interest to develop non-invasive methods to determine the sex of pre-implantation embryos. One successful example is the production a male mouse line with a labeled X chromosome (9).

It is also of interest to develop methods to improve the efficiency of generating transgenic animals, especially livestock. Genetically modified livestock are produced to satisfy the need for food or non-food products (10–12). However, whereas genetically modified mice can be easily obtained through genetic modification of embryonic stem cells (ESCs) or direct embryo injection, isolating and culturing ESCs for livestock is difficult and pronuclear microinjection is less efficient (13, 14). Therefore, the most popular method for producing genetically modified livestock is somatic cell nuclear transfer (SCNT) (15–17). The major advantage of SCNT over direct embryo injection is the predictable genotype of the offspring and the ability to generate clonal lines of edited animals (18). A variety of transgenic livestock models have been produced with improved growth rate, resistance to disease, and more efficient feed usage using somatic cell cloning techniques (19–21).

Unlike embryos derived from fertilization, most cloned embryos die before and after implantation, and even those that can survive to term are frequently defective, vulnerable to miscarriage, high birth weight or perinatal death (22–26). DNA methylation, histone methylation and histone acetylation are closely related to the developmental potential of cloned embryo in mammals (27, 28). Histone acetylation is an important event in epigenetics, and reprogramming of histone acetylation in donor cells is very important for turning the nucleus of a highly differentiated state into a pluripotent state. HDACs are histone deacetylases, hypoacetylated histones are related to lower transcriptional activity due to closed chromatin structure formation (29). There are considerable evidences to suggest that the abnormal epigenetic reprogramming of cloned embryos is an important cause for its low development potential (30–32). Studies have shown that the DNA methylation status of donor cells can also affect the efficiency of nuclear reprogramming, and the level of DNA methylation is inversely related to the developmental potential of cloned embryos (33, 34). DNMT1 and DNMT3a are DNA methyltransferases (35), responsible for the maintenance of DNA methylation and the establishment of *de novo* methylation during early embryonic development. The lower the DNA methylation level of the donor cell, the higher the embryonic development ability (36–39), and reducing the DNA methylation level of donor cells can improve the developmental efficiency of cloned embryos (36, 40–42). However, some studies have found that 5-aza-2'deoxycytidine (5-aza-dC), a DNA demethylation drug, cannot effectively improve the development efficiency of cloned embryos (43–46). This result indicates that the relationship between the DNA methylation status of donor cells and the development efficiency of cloned embryos needs to be further clarified. The efficiency with which transgenic embryos are created using SCNT is mainly determined by the ability to obtain genetically modified somatic cells. Many types of somatic animal cells can be used as donor cells for SCNT (47–49). Fetal fibroblasts have become widely used to produce transgenic livestock for several advantages. For example, they are easy to obtain and culture, and can be genetically modified and develop into live offspring. However, the low efficiency of introduction and integration of exogenous DNA into the genomes of primary mammalian cells, such as fetal fibroblast cells, has greatly limited the production of transgenic animals by SCNT. Other factors that may affect the efficiency of transgenic animal production efficiency are DNA methylation and histone acetylation, which are involved in remodeling the genome and are linked to gene imprinting in the early embryo, and cell viability.

One major method used to integrate exogenous DNA into the genome is homology-directed repair (HDR), nevertheless, the efficiency of HDR is very low (<1%) without selection (50–52). To overcome this limitations, researchers have employed the type II bacterial clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system, which has been engineered into an efficient genome-editing tool consisting of the Cas9 nuclease and a single guide RNA (sgRNA) (53, 54). At present, CRISPR/Cas9 mediated HDR is the commonly used approach to achieve precise and targeted integration of transgenes because of its high efficiency. There are some reports that the length of the homology arm (HA) can dramatically influence the efficiency of targeted integration of exogenous genes (55, 56). Therefore, it may be possible to further improve the efficiency of CRISPR/Cas9-mediated HDR in the cells by optimizing HA length.

The integration of exogenous DNA into genome is based on DNA damage and repair by the CRISPR/Cas9 system. Severe deleterious consequences of Cas9-induced DNA damage, mainly DNA double-strand breaks (DSBs), have been observed in some studies (57, 58). What's more, some researchers have found that a single targeted DNA break is enough to cause cell cycle arrest or apoptosis in some cell types (59, 60). Further experimental evidence in human pluripotent stem cells showed that introduction of DSBs at a single locus is sufficient to cause a P53-dependent molecular response and that transient suppression of the activity of p53 can improve the efficiency of precise genome engineering (60). Transient suppression of p53 activity can be achieved by treating cells with Pifithrin-μ (PFT-μ), a small molecule that inhibits binding of p53 to mitochondria by reducing its affinity to the anti-apoptotic proteins Bcl-xL and Bcl-2 but has no effect on p53-dependent transactivation (61).

Guangxi province has the largest number of buffalo in China, and is the main force driving the development of China's

dairy buffalo industry. Increasing the number and improving the quality of dairy herds are crucial ways to accelerate the development of Guangxi's dairy buffalo industry. Here, we used buffalo as a research model for optimizing two factors that limit the genetic modification of somatic cells: the introduction of exogenous DNA and the integration of the DNA into the genome. Specifically, we optimized the parameters for introduction of exogenous DNA into buffalo fetal fibroblast (BFF) cells using electroporation, which is one of the most widely used methods for introducing exogenous DNA into somatic cells because of its high efficiency. We also optimized methods to improve the efficiency of exogenous gene integration in BFF cells using the CRISPR/Cas9 system. Our previous research revealed that a labeled Y chromosome is suitable for the identification of the sex of pre-implantation mouse embryos (62). In this study, we developed a Y chromosome-linked eGFP mouse line that stably expresses green fluorescent protein under the control of the CAG promoter. A tracer to the Y chromosome has simplified the process of predicting the sex of embryos collected by mating a Y-Chr-eGFP transgenic male with a wild-type female, all XY embryos appeared green, under a fluorescence microscope, and XX embryos did not. The accuracy of this method can reach 100%, which provides an expeditious and accurate approach for sexing pre-implantation embryos. To confirmed the applicability of this method in livestock, buffalo served as a model. We successfully established procedures for producing transgenic cloned buffalo embryos with the *eGFP* gene integrated at a specific locus on the Y chromosome using the CRISPR/Cas9 system combined with SCNT. We then compared Y-Chr-eGFP transgenic cells and non-transgenic BFFs in terms of various parameters related to cell growth and proliferation, and expression level of epigenetic-related genes. Finally, we compared the developmental competence of embryos produced by cloning using Y-Chr-eGFP transgenic and non-transgenic BFFs as donor cells.

MATERIALS AND METHODS

All animal experimental protocols were performed in accordance with the relevant ethical guidelines and regulations. Buffalo ovaries used for the *in vitro* production of mature oocytes used as SCNT recipients were collected from a slaughterhouse in a suburban area near Nanning City, China.

Unless otherwise stated, all organic and inorganic reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Self-made solutions were filtered through a 0.22-μm filter (Millipore, Bedford, MA, USA) and stored at 4°C or at −20°C until use. Pipette tips, centrifuge tubes and petri dishes were purchased in aseptic packages and were all disposable.

Construction of the CRISPR/Cas9 Plasmid and the Homologous Recombination Repair Vector

Design and construction of the CRISPR/Cas9 plasmid were performed based on the method described by Ran et al. (63). An sgRNA targeting intron 12 of Ddx3y (Gene ID: 783057) was designed using the CRISPR design tool (http://tools.genome-engineering.org). Plasmid px459 (Addgene, catalog no. 62988) was linearized by digestion with the BbsI restriction enzyme (NEB catalog no. R0539S) and ligated with the annealed sgRNA using T4 DNA ligase (Thermo Fisher Scientific, catalog no. EL0011). DNA sequencing was performed to confirm the accuracy of the fully constructed CRISPR/Cas9 plasmid.

To construct the homologous recombination (HR) donor, CAG-EGFP, the 5′ and 3′ HA sequences were amplified from CAG-GFP-IRES-CRE (Addgene, catalog no. 48201) or the animal genome, then subcloned into the donor vector by restriction enzyme digestion and ligation.

The Design and Screening of sgRNAs

The RFP-GFP-surrogate (RGS) reporter system was used for the screening of sgRNAs (64). This system consists of a red fluorescent protein (RFP) and the 20 bp target sequence plus a 3-bp PAM sequence and a GFP cassette. However, the DNA sequence of GFP is out of reading frame, the reading frame of GFP gene may be recovered by repair of DNA damage when the target sequence ahead of GFP gene is cut. The RGS plasmid was linearized by EcoRI and BamHI. Then we inserted the sgRNA with the PAM into the linearized plasmid. The RGS and px459 plasmid carrying the sgRNA were cotransfected into HEK 293T cells. The transfected cells expressed RFP, and the cleavage rate of sgRNA was measured by determining the percentage of cells expressing GFP.

Cell Culture and Transfection

Mouse ESCs were cultured in 2i medium, which consists of Dulbecco's modified Eagle's Medium (DMEM) (Gibco), supplemented with 10% fetal bovine serum (FBS), 1,000 U/mL mouse Lif, 2 mM glutamine (Sigma), 1% penicillin/streptomycin (Thermo Fisher Scientific), 0.1 mM non-essential amino acids (Gibco), 1 μM PD0325901 and 3 μM CHIR99021. Mouse Neuro-2a (N2a) and 3T3 cells were cultured in DMEM/F12 (Gibco) containing 10% FBS, 1% penicillin/streptomycin (Solarbio), and 0.1 mM non-essential amino acids (Gibco).

All cells were cultured at 37 °C in a humid atmosphere of 5% CO_2. Transfection of the cells (mES, N2a, and NIH3T3) was performed using Lipofectamine 3000 Reagent (Invitrogen) according to the manufacturer's instructions. Plasmids containing sgRNA, Cas9 and a donor were transfected into the cells, and 48 h later, transfection-positive cells were sorted by flow cytometry for further analysis.

Preparation of Buffalo Fetal Fibroblasts

Buffalo fetuses aged 3–4 months were taken from a local slaughterhouse and transported back to the laboratory within 2 h. The amniotic membrane of the fetus was removed with tweezers, and the fetus was washed two to three times in phosphate-buffered saline (PBS) containing penicillin and streptomycin. After washing, the skin tissue was cut into small pieces with sterilized ophthalmic surgical scissors, then placed in a culture plate with a small amount of culture medium and kept in a cell incubator. After culturing for 72 h, the tissue blocks were removed and the cells were continuously cultured for another 2

days, when the adherent cells approached confluence, then stored in liquid nitrogen.

Optimizing Voltage and Pulse Number to Maximize Delivery Efficiency of Exogenous DNA Into Buffalo Fetal Fibroblast Cells

To examine the effect of voltage on the efficiency of plasmid delivery to fibroblast cells, 20 μg purified vector DNA was mixed into 100 μl PBS containing 1.5×10^6 cells. A single pulse of 0, 100, 150, 200, 250, or 300 V for 1 ms was administered to the BFF cells. Following electroporation, the cells were resuspended in 3 ml of culture media and seeded on a 60-mm culture dish. Thirty-six hours after transfection, each well was washed with PBS and fresh medium containing Hoechst 33258 was added. Plates were then incubated for 20 min at 37°C. The total number of cells in each well was counted based on Hoechst nuclear staining visualized under a fluorescence microscope, and the ratio of the cells expressing eGFP in a field of view (the counts from more than five fields of view were averaged) was determined. Survival rate was defined as the total number of cells divided by the number of cells in the 0 V group in one field of view (an average of five fields were observed per well).

Based on the results of a single pulse at multiple voltages, 250 V was chosen to evaluate the effect of multiple pulses. BFF cells were subjected to 1, 2, 3, 4, or 5 pulses (1 ms each) of 250 V, and 36 h after electroporation, electro-transfection efficiency was analyzed.

Transfection and Selection of Gene-edited Cell Colonies

Two days before electroporation, the frozen cells were thawed and seeded in a 60-mm cell culture dish (Axygen), and transfection was performed only when confluence reached 70–85%. For electroporation to introduce DNA into somatic cells, three 1-ms pulses of 250 V were applied to 100 μl of 2×10^6 cells in the presence of 10 μg px459 vector and 10 μg donor plasmid. For electroporation, square wave pulses were administered through a BTX ECM 2001 in 2-mm gap cuvettes. Thirty-six

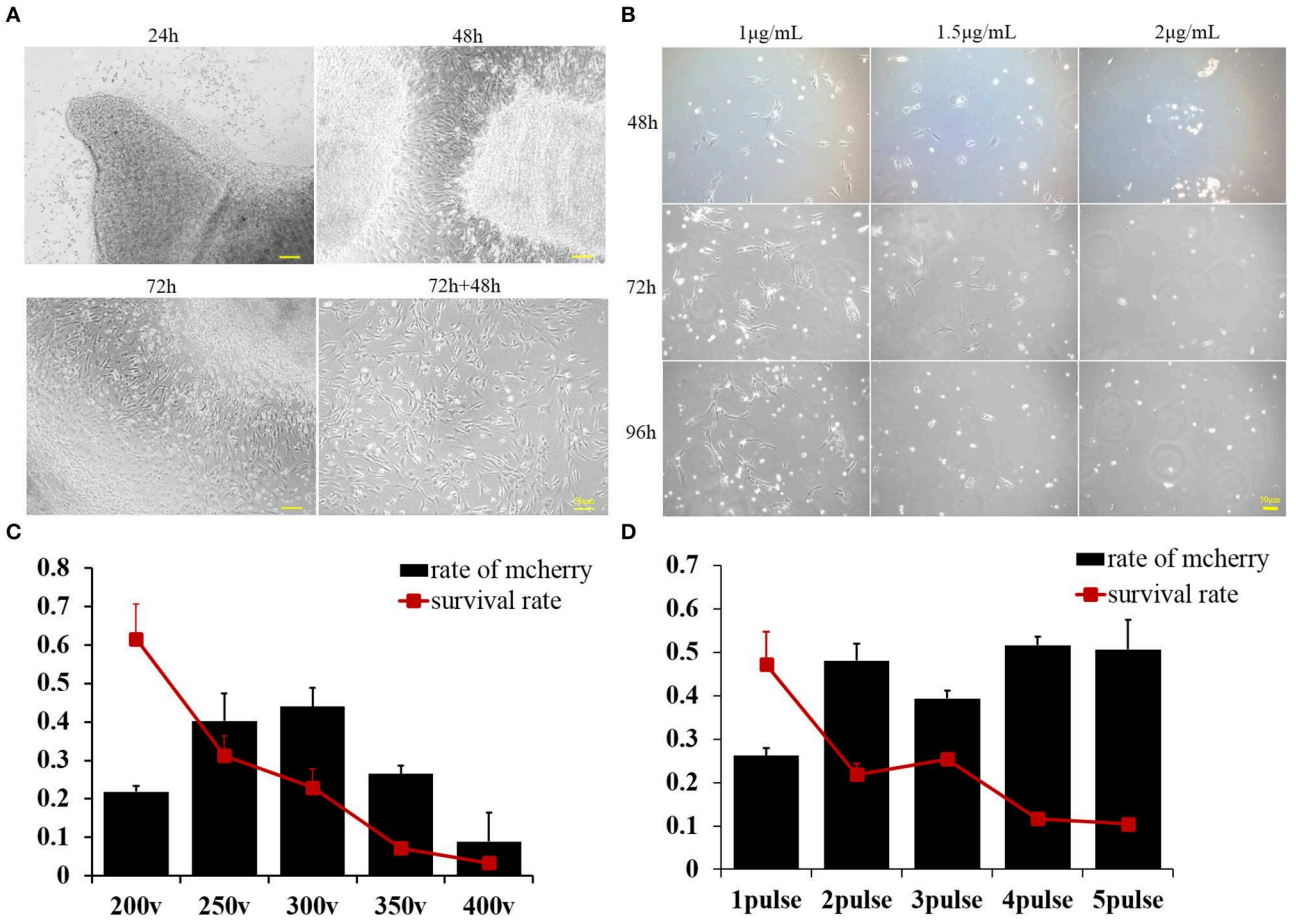

FIGURE 1 | (A) Buffalo fetal fibroblast cells were isolated and cultured using the explant technique for 72 h, then continuously cultured for another 2 days after removing the minced tissues. Pictures were taken at 24, 48, and 72 h after culturing and again 48 h after removing the minced tissues. **(B)** Buffalo fetal fibroblast cells were cultured in the presence of different concentrations of puromycin, and pictures were taken after 48, 72, and 96 h. **(C)** The percentage of transfected cells as determined by mCherry expression was affected by voltage. Cell survival was also greatly affected by the voltage. **(D)** The percentage of cells effectively transfected with supercoiled vector and expressing mCherry, was affected by the number of pulses. Cell survival was also greatly affected by the pulse number. Scale bar = 50 μm.

hours later, the media was changed to introduce 1.5 μg/mL puromycin (Solarbio, Beijing, China). Cells were incubated for 4 days, and individual cell colonies formed after withdrawing puromycin were continuously cultured for an additional 7–10 days. Individual colonies expressing the GFP genes under fluorescence microscope were picked up and cultured in a 96-well cell culture plate. When confluence was achieved, the cell colonies were sub-cultured to obtain cells for genotyping and SCNT.

The Genotyping Analysis of the Cells Expressing GFP Genes

Single cell clones expressing green fluorescence were expanded and cultured for genotyping, and primers were designed to the identify the accuracy of integration junction sites. 5′ and 3′ junction sites of exogenous gene were amplified by PCR, the primers used for amplification of 5′ and 3′ junctions were listed in **Table S3**, then sequenced the PCR products of clones which were double positive at 5′ and 3′ junctions. Choosing the clones that were precise integrations as nuclear transfer donor cells.

The Expression Level of Epigenetic Related Genes

The expression level of epigenetic related genes was analyzed by real-time quantitative PCR. Total RNA was isolated from BFF and Y-Chr-eGFP transgenic BFF cells using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's

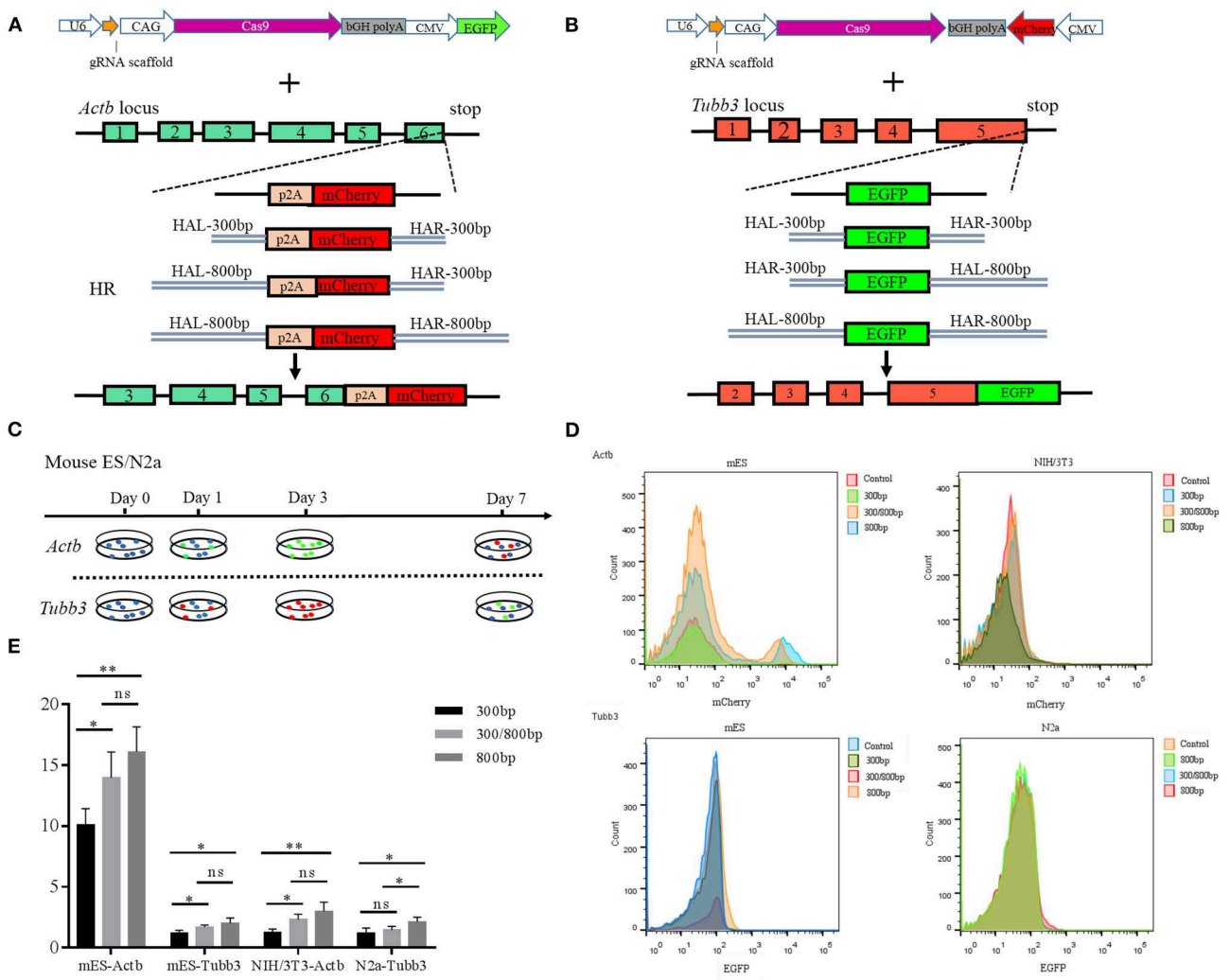

FIGURE 2 | (A) Schematic overview of the strategy used to target the *Actb* locus. HAL/HAR, left/right homology arm; HR, homologous recombination; **(B)** Schematic overview of the strategy used to target the *Tubb3* locus; **(C)** Experimental scheme for targeted knock-in of *Actb-p2A-mCherry* in mouse ES and NIH/3T3 cells and *Tubb3-EGFP* in mouse ES and N2a cells. Cells were transfected with donors/GFP or donors/mCherry and Cas9/sgRNA/mCherry/GFP, and transfected cells were sorted based on GFP or mCherry signals 2 days after transfection. **(D)** Knock-in efficiencies were evaluated by fluorescence activated cell sorting (FACS) and were based on the ratio of GFP$^+$ or mCherry$^+$ cells to all transfected cells; **(E)** Histograms show relative knock-in efficiencies for different homology arm lengths used for knock-in at *Actb* and *Tubb3* in mouse cells expressed as the percentage of mCherry$^+$ (or GFP$^+$) cells among all transfected cells. Results were presented as mean ± s.d. $^*P < 0.05$, $^{**}P < 0.01$, ns, no significant difference, unpaired Student's *t*-test.

instruction. The cDNA was obtained from ∼1 μg RNA and reverse transcribed by the PrimeScriptTM RT Reagent Kit with gDNA Eraser (Takara, Japan). Real-time quantitative PCR were performed using CFX96 C1000 Thermal Cycler (Bio-Rad, USA) by TB Green® Premix Ex Taq™ (Tli RNaseH Plus) (Takara, Japan) in triplicate. PCR was performed using two steps for 40 cycles at 95°C for 5 s, 60°C for 30 s and followed by melt curve. All the gene expression levels were normalized to the internal standard gene Gapdh. Relative gene expression was determined using $2^{-\Delta\Delta Ct}$ method. The primer sequences are listed in **Table S4**.

Cell Cycle and MTT Assay

BFF cells were incubated in the presence of PFT-μ for additional 24 and 48 h, subsequently, PI and RNase A were added the collected cells for 30 min of incubation at 37°C. Cells were analyzed by flow cytometry (BD Biosciences) and data analysis was performed using Flow Jo 10.

Cell viability was assessed using the MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Shanghai, China). Briefly, BFF cells were digested and seeded into 96-well plates at a density of 2×10^3 cells per well. Next, cells were incubated with MTT solution for 4 h at 37°C. Formazan solution was subsequently added to the 96-well plates to dissolve the formazan crystals that formed. The optical density (OD) was measured at 570 nm.

Production of Y-Chr-eGFP Buffalo Embryos via SCNT

SCNT was performed as described previously (65–68). Briefly, gene-edited fibroblasts were thawed and cultured in serum starvation conditions (DMEM/F12 supplemented with 0.5% FBS) for 2 days to synchronize the cell cycle. The cells were harvested and resuspended with micromanipulation medium (10 mM HEPES-buffered TCM-199 containing 0.3% [w/v] bovine serum albumin; pH = 7.3). Cumulus-oocyte complexes were aspirated from the follicles and cultured in preheated maturation medium (bicarbonate-buffered TCM-199 supplemented with 5% estrous bovine serum and 10 μg/mL follicle-stimulating hormone [FSH]). The oocytes were cultured in an incubator at 39°C under 5% CO_2 for another 22–24 h. Oocytes with an easily defined first polar body and homogenous cytoplasm were selected for use as nuclear transfer recipients.

Matured oocytes were enucleated by aspirating the first polar body plus a portion of the adjacent cytoplasm, then the donor cell nucleus was injected into the perivitelline space. The fusion of nuclear-transferred embryos was performed using the following program: two successive DC pulses of 2.1 kV/cm for 30 μs under a BTX ECM 2001 electro cell manipulator. Three hours after the fusion, the activation of reconstructed embryos was induced by exposure to 5 μM ionomycin in embryo culture medium for 5 min and subsequent incubation in 2 mM 6-dimethylaminopurine for 4 h at 39°C and 5% CO_2.

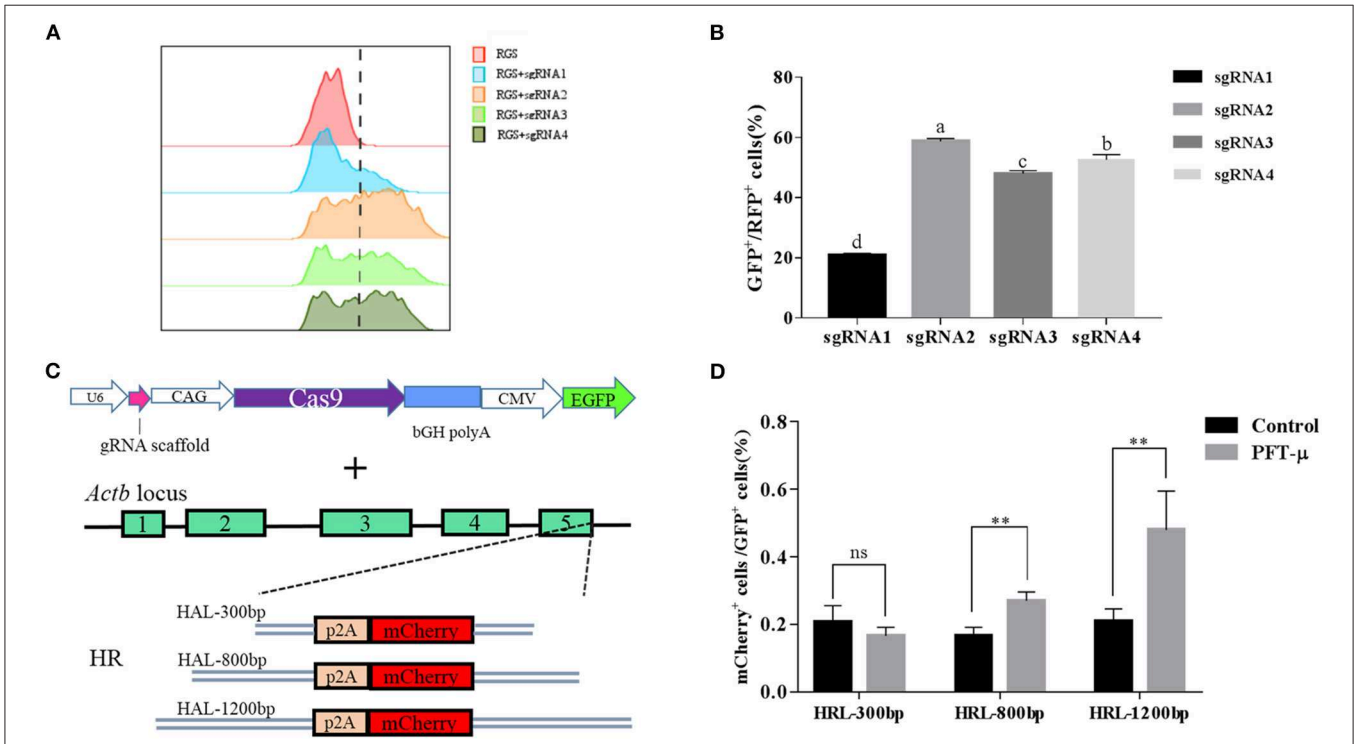

FIGURE 3 | (A) The RGS reporter system was used to determine the efficiencies of sgRNAs; the cleavage activities were evaluated by FACS and are presented as the ratio of GFP+/RFP+ cells to all transfected cells; **(B)** Histograms show the relative cleavage activity of different sgRNAs; **(C)** Schematic overview of the strategy used to target the *Actb* locus in buffalo fetal fibroblast cells; **(D)** Knock-in efficiencies were evaluated by FACS and are presented as the ratio of mCherry+ cells to all transfected cells. $^{**}P < 0.01$, ns, no significant difference.

After activation, reconstructed embryos were placed in IVC medium (modified Tyrode's medium supplemented with 36% TCM-199, 10% FBS, 0.06 mg/mL penicillin and 0.1 mg/ml streptomycin) in a 20-μl culture droplet overlaid with mineral oil under a humidified atmosphere of 5% CO_2 at 39°C. The granulosa cell monolayers were established at least 12 h before the introduction of embryos. The culture medium was refreshed every 48 h after the introduction of embryos by replacing half of the original medium with a similar volume of fresh medium.

The Development of Cloned Embryos From Y-Chr-eGFP Transgenic BFF Cells

Embryonic cleavage rate and blastocyst formation rate were two parameters we monitored during *in vitro* embryonic development. The blastocyst rate was recorded on day 8 of IVC. Cleavage rate and blastocyst formation rate between BFF cells and Y-Chr-eGFP transgenic BFF cells were analyzed on experiments repeated five times using one-way ANOVA.

Statistical Analysis

The data are presented as the mean±SEM. At least three replicates were tested for each group. Statistical analyses were performed using Prism 7 software (Graph Pad, San Diego, CA, USA), using analysis of variance (ANOVA) and Student's *t*-tests where appropriate.

RESULTS

Preparation of Buffalo Fetal Fibroblast Cells and Optimization of the Concentration of Puromycin

BFF cells were derived from buffalo fetal skin using the direct adherent culture method. Tissue pieces were seeded in a culture dish, and fibroblasts derived from the minced tissues were observed with increasing incubation time (**Figure 1A**). Seventy-two hours later, tissue pieces were removed from the dishes and cultured for another 2 days until the fibroblasts reached 70–80% confluence (**Figure 1A**). Cells were then frozen in liquid nitrogen until further use. To optimize the concentration of puromycin, the culture medium was supplemented with increasing doses of puromycin (1, 1.5, and 2 μg/mL). Almost all of the BFF cells treated with 1.5 μg/mL puromycin for 4 days died (**Figure 1B**). Therefore, 1.5 μg/mL was determined to be the minimum concentration that can be used for screening BFFs carrying puromycin resistant gene.

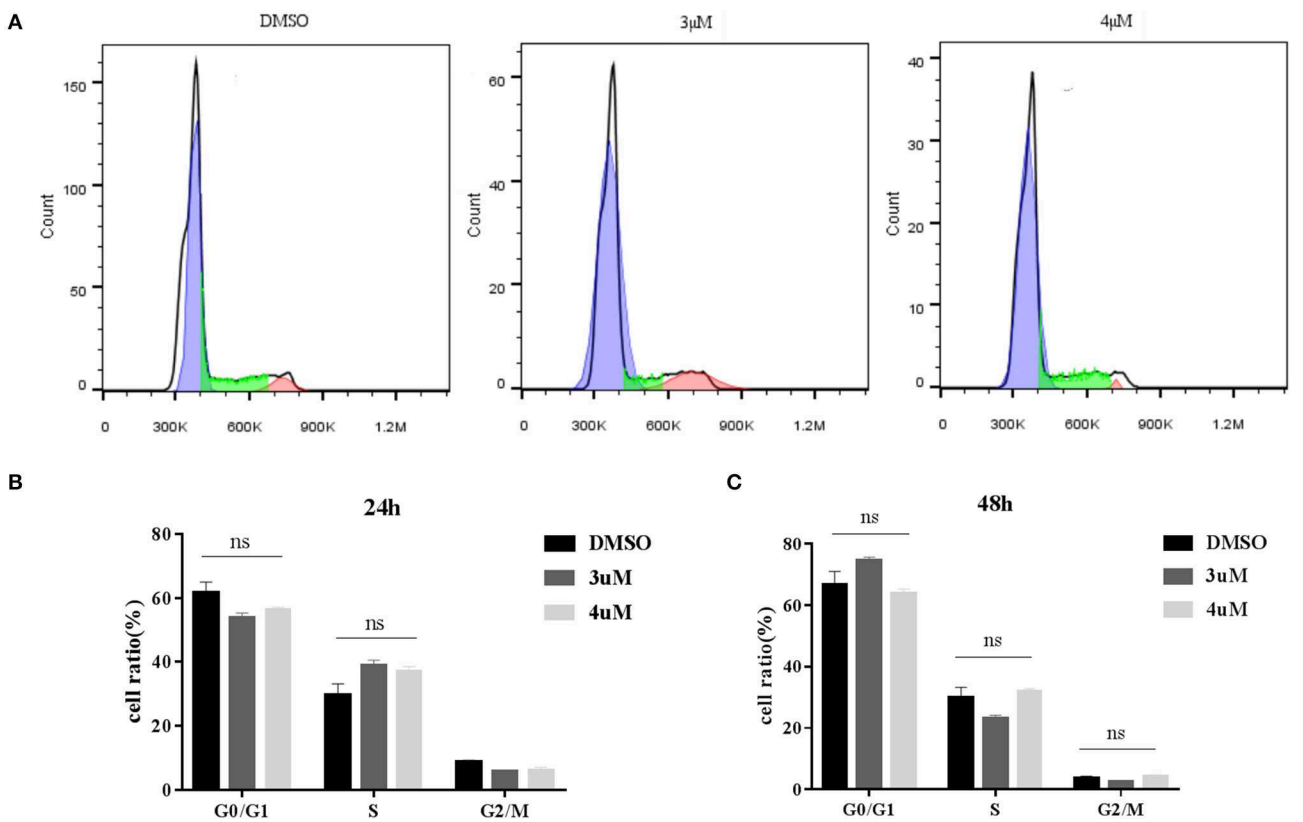

FIGURE 4 | (A) Cell cycle of BFF cells with different concentration of PFT-μ; **(B)** Histograms show the change of cell cycle in BFF cells with different concentration of PFT-μ for 24 h; **(C)** Histograms show the change of cell cycle in BFF cells with different concentration of PFT-μ for 48 h.

Optimization of the Voltage and Pulse Number Used to Deliver Exogenous DNA Into Buffalo Fetal Fibroblasts

A plasmid containing a mCherry reporter gene with expression driven by the CAG promoter was used to estimate the cell transfection efficiency. The percentage of transfected cells was determined by monitoring the expression of mCherry in the cytoplasm of cells. We concluded that voltage can greatly affect the transfection efficiency, with higher voltages producing more transfected cells; however, cell survival decreased with increasing voltage delivered as a single pulse (**Figure 1C**). Given that 250 V maximized both transfection efficiency and cell survival, this voltage was used to further optimize the number of pulses. According to our second experiment, we deemed that two pulses can achieve the maximum delivery of exogenous supercoiled vector DNA into BFFs without compromising survival (**Figure 1D**).

The Length of the Homology Arm Affects the Efficiency of CRISPR/Cas9-mediated Homologous Recombination

We next determined whether the length of the HA affects the knock-in efficiency when using an HR-based method. We constructed three donor plasmids with HAs of 300 bp on both sides of the target, 300 bp on one side and 800 bp on the other, and 800 bp on both sides. To assess the knock-in efficiencies, we aimed to fuse a *p2A-mCherry* gene to the last codon of the *Actb* gene in the mouse cell lines ES and NIH3T3, and an *eGFP* reporter gene to the last codon of the *Tubb3* gene in the mouse cell lines ES and N2a (**Figures 2A,B**). The experimental scheme for targeted *Actb-p2A-mCherry* and *Tubb3-EGFP* knock-in in mouse ES, NIH3T3, and N2a cells is shown in **Figure 2C**. Seven days after transfection, the knock-in efficiencies for HAs of different lengths were evaluated by flow cytometry and are presented as the percentage of cells expressing the specific reporter protein (**Figures 2D,E**). We found the highest knock-in efficiency when using an HA of 800 bp for all three types of cell lines.

PFT-μ Can Improve the Efficiency of Exogenous Gene Integration Mediated by CRISPR/Cas9

We next determined whether addition of PFT-μ to the culture medium affects the knock-in efficiency when using the HR-based method. We constructed three donor plasmids with HA

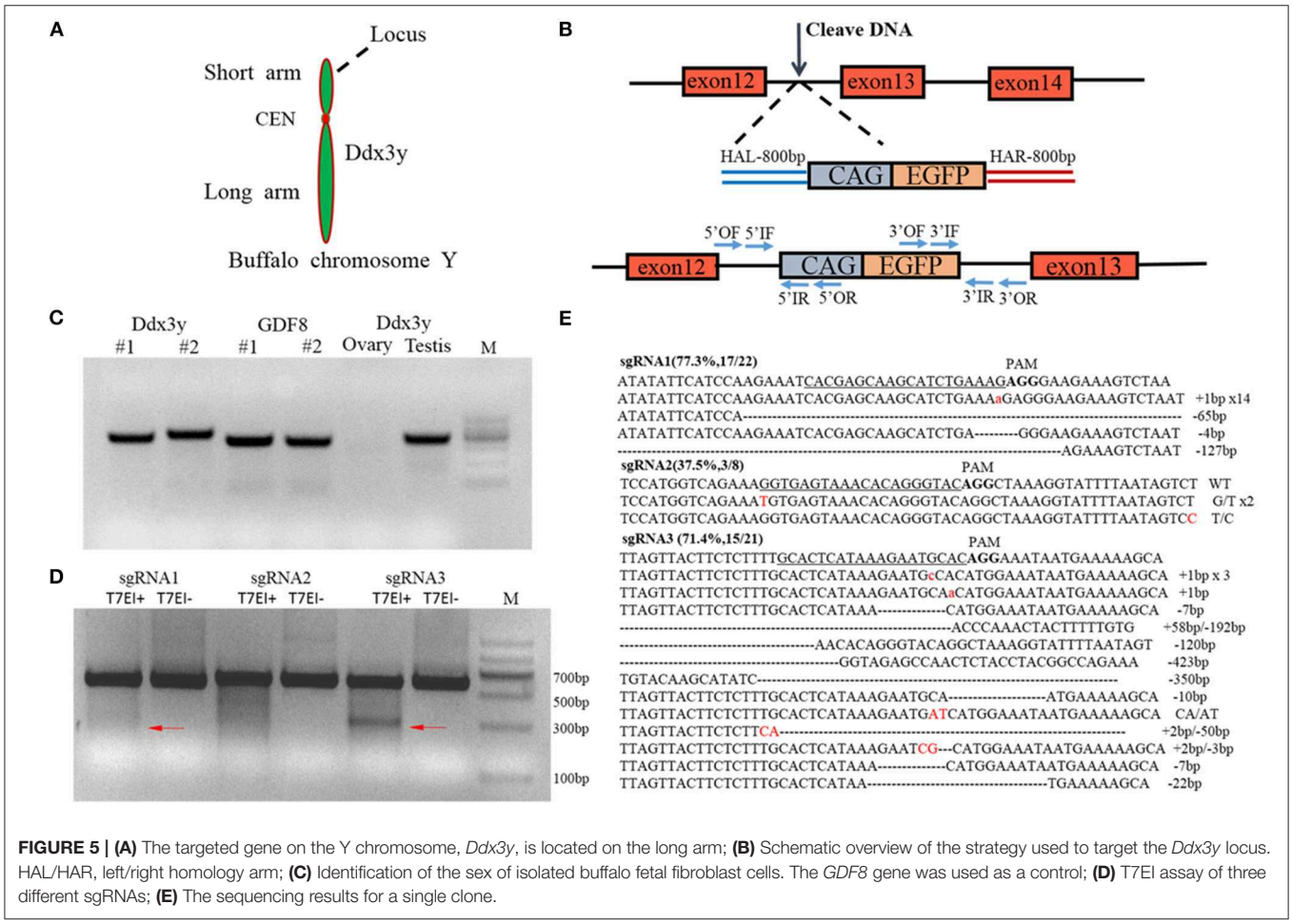

FIGURE 5 | (A) The targeted gene on the Y chromosome, *Ddx3y*, is located on the long arm; **(B)** Schematic overview of the strategy used to target the *Ddx3y* locus. HAL/HAR, left/right homology arm; **(C)** Identification of the sex of isolated buffalo fetal fibroblast cells. The *GDF8* gene was used as a control; **(D)** T7EI assay of three different sgRNAs; **(E)** The sequencing results for a single clone.

lengths of 300, 800, and 1,200 bp. To assess the knock-in efficiencies, we aimed to fuse a *p2A-mCherry* reporter gene to the last codon of the β-*actin* gene in BFF cells. We designed four sgRNAs targeting the defined locus (**Table S2**), and the results from the RGS reporter system showed that sgRNA2 was the most efficient; therefore, this sgRNA was selected for subsequent flow cytometry experiments (**Figures 3A,B**). A schematic overview of the strategy used to target the *Actb* locus in BFF cells is shown in **Figure 3C**. Seven days after transfection, the knock-in efficiencies for HAs of different lengths in the presence of PFT-μ were evaluated and are presented as the percentage of cells expressing mCherry, as determined by flow cytometry (**Figure 3D**). Higher knock in efficiencies in BFF cells were observed with an HA length of 1,200 bp, and PFT-μ significantly improved the HR efficiency when the HA length was 800 or 1200 bp (**Figure 3D**). Cells cycle were analyzed after incubated with different concentrations of PFT-μ for 24 or 48 h, the results indicate that no significant differences in cell cycle with the presence of PFT-μ or not (**Figure 4**).

Establishment of Y-Chr-eGFP Transgenic Cells

A construct was designed to integrate the *eGFP* gene into intron 12 of the *Ddx3y* gene (Gene ID: 783057) located on Y chromosome in BFFs as shown in **Figures 5A,B**. The sex of harvested BFF cells was determined by amplifying *Ddx3y*, and the *GDF8* gene located on an autosome was amplified as a control. The results of sex identification showed that male BFFs were successfully obtained (**Figure 5C**). Three sgRNAs were designed to target the intron of the *Ddx3y* gene (**Table S1**), and based on the combined results of T7EI (**Figure 5D**) and sequencing (**Figure 5E**), sgRNA3 was chosen for further experiments. Px459 and an HR donor carrying a CAG promoter, an *eGFP* gene, and 5′ and 3′ homologous sequences were co-transfected to BFFs. A schematic overview of the strategy used to target the *Ddx3y* locus is shown in **Figure 5B**. Screening for Y-Chr-eGFP transgenic cells was then performed. Only a few cells expressing the puromycin gene survived in the presence of puromycin for 4 days. After puromycin was removed and cells were cultured for 7–10 days, individual eGFP-positive cell colonies were recovered (**Figure 6A**). We randomly picked 42 puromycin-resistant eGFP-positive cell colonies for proliferation, and genotyped the expanded cell colonies using PCR and DNA sequencing to confirm that the desired gene integration had occurred (**Figure 6B**). The desired integration was only found in 9.2% of the colonies (**Figure 6C**). The results of PCR and DNA sequencing of these colonies also confirmed that fragments of the expected sizes, 998 bp for the 5′ junction and 1,101 bp for the 3′ junction, were amplified, indicating that the cells contained the correctly integrated *eGFP* gene (**Figures 6D,E**).

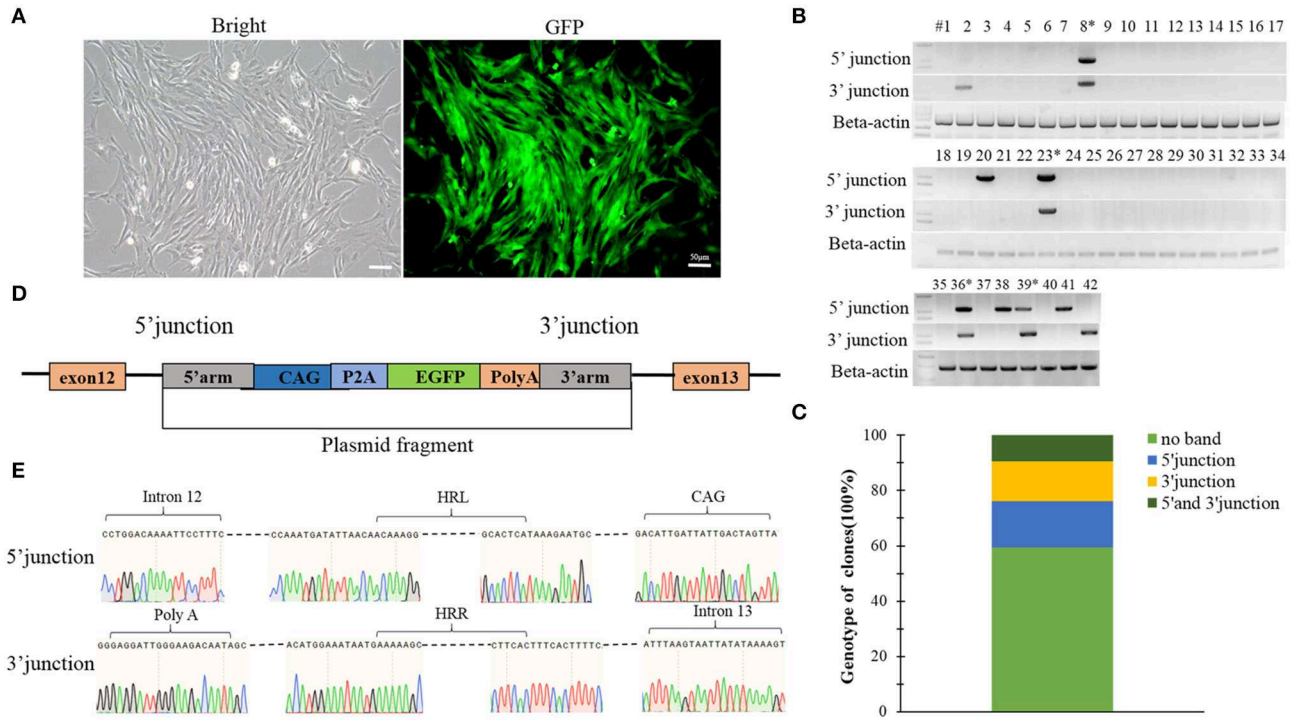

FIGURE 6 | (A) A single puromycin resistant colony cultured for 7 days after withdrawing puromycin; **(B)** Screening for the integrated transgene in all 42 selected colonies; **(C)** The percentage of the 42 colonies with a given genotype (no integration, only 5′ junction, only 3′ junction or both 5′ and 3′ junctions); **(D)** Schematic overview of the strategy used to target the *Ddx3y* locus; **(E)** Sequences of PCR products amplified from the 5′ and 3′ junctions. Scale bar = 50 μm.

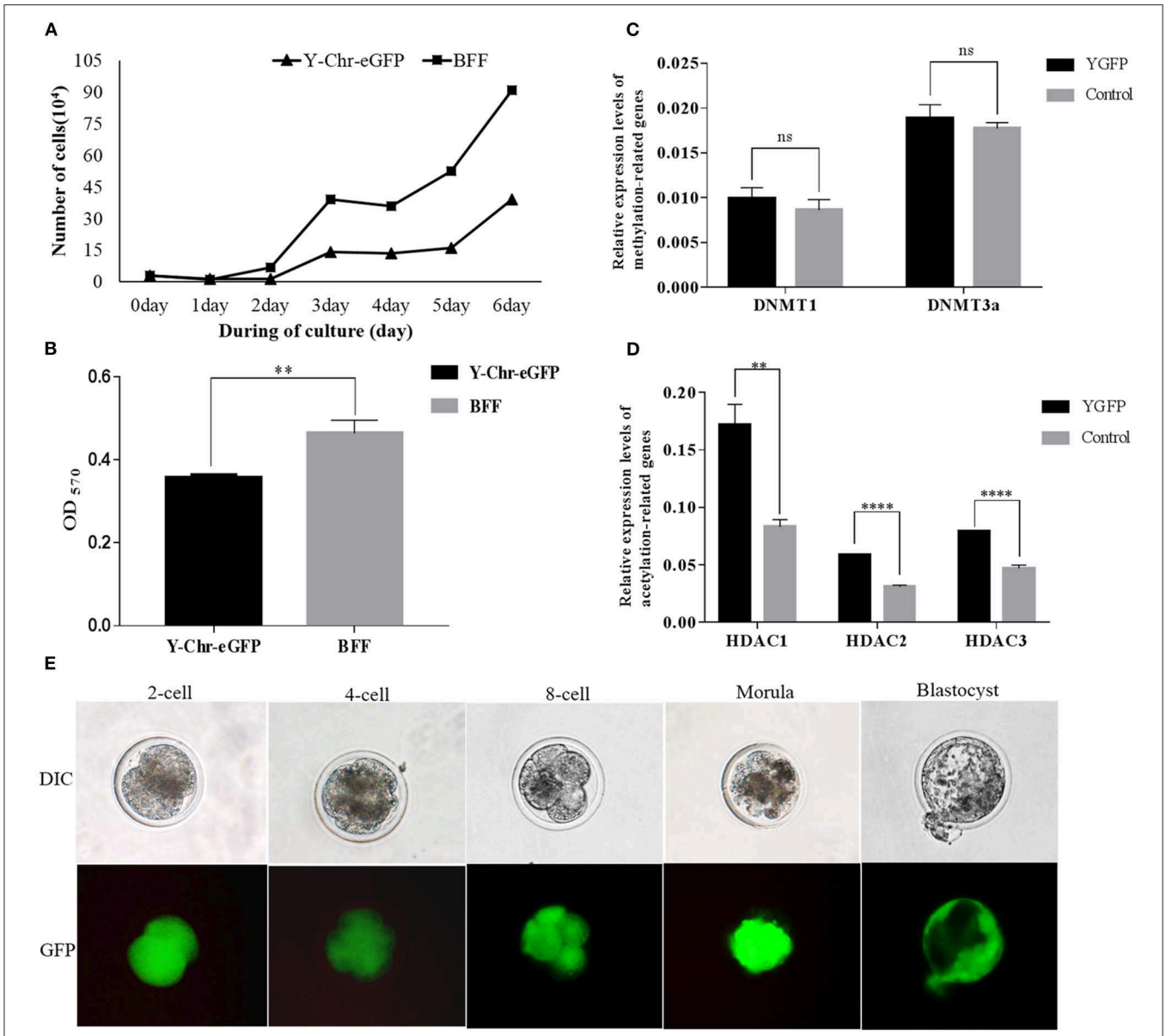

FIGURE 7 | (A) Proliferation of Y-Chr-eGFP transgenic and non-transgenic buffalo fetal fibroblast cells grown for 144 h in a 6-cell dish; **(B)** Cell viability of Y-Chr-eGFP transgenic and non-transgenic buffalo fetal fibroblast cells evaluated by MTT assay; **(C)** Relative expression levels of DNA methylation-related genes in Y-Chr-eGFP transgenic and non-transgenic fetal fibroblast cells determined by real-time PCR. **(D)** Relative expression levels of histone acetylation-related genes in Y-Chr-eGFP transgenic and non-transgenic fetal fibroblast cells determined by real-time PCR. Values are reported as the mean ± SEM; $^{**}P < 0.01$, $^{****}P < 0.0001$. **(E)** Y-Chr-eGFP transgenic embryos derived from SCNT at different stages of development.

Comparison of the Relative Cell Viability and Proliferation Rate of Y-Chr-eGFP Transgenic and Non-transgenic Cells

The proliferation rate of Y-Chr-eGFP transgenic cells was found to be significantly lower than that of non-transgenic cells (**Figure 7A**). Also, the relative cell viability determined by MTT assay was significantly lower for Y-Chr-eGFP transgenic cells compared with non-transgenic cells (**Figure 7B**).

Analysis of Epigenetic-Related Gene Expression in Y-Chr-eGFP Transgenic and Non-transgenic Cells

Comparison of the relative gene expression levels of methylation-related genes in the Y-Chr-eGFP transgenic and non-transgenic cells revealed that the expression levels of *DNMT1* and *DNMT3a* were similar between the two groups (**Figure 7C**). However, the expression levels of the acetylation-related genes, *HDAC1*, *HDAC2*, and *HDAC3*, were significantly

higher in Y-Chr-eGFP transgenic cells than in non-transgenic cells (**Figure 7D**).

Developmental Competence of Y-Chr-eGFP Transgenic and Non-transgenic Embryos

Cloned Y-Chr-eGFP buffalo embryos produced using donor cells with well-defined genotypes, showed strong expression of the eGFP gene during embryo development *in vitro* (**Figure 7E**). This demonstrates that Y-Chr-eGFP transgenic fibroblasts used as donor cells could be successfully reprogrammed and form into blastocysts *in vitro*. The rates of development of cloned embryos produced from Y-Chr-eGFP transgenic and non-transgenic cells are in presented in **Table 1**. The cleavage rate was significantly higher ($P < 0.05$) for embryos produced from non-transgenic cells than for those derived from Y-Chr-eGFP transgenic cells.

TABLE 1 | Developmental competence of cloned embryos produced from transgenic and non-transgenic fetal fibroblast cells.

Donor cell type	Reconstructed embryos (*n*)	Cleaved embryos *n* (%)	Blastocysts *n* (%)
Fetal fibroblast cells	199	71 (36.01+0.42)[a]	9 (13.55+1.11)[a]
Y-Chr-eGFP transgenic fetal fibroblast cells	242	76 (30.94+1.56)[b]	10 (13.45+0.84)[a]

Blastocyst rate data are from 5 trials. Values are mean ± SEM. Values with different superscript within the same column differ significantly ($p < 0.05$).

DISCUSSION

Genetically modified animals play a remarkable role in agriculture and biomedicine (69–72). Limitations in the efficiency of genetic modification of primary mammalian cells including fibroblasts greatly limits the production of transgenic animals. Many attempts have been made to optimize the efficiency of integration of exogenous DNA into genomes (73–75). HR is one of the most important method to conduct precise gene editing (73), which is generally inefficient in mammalian cells for that HR pathway functions during the late S/G2 phase (76, 77). However, DSBs can stimulate the HR pathway (76) and HR requires the presence of homologous sequences (78, 79), using CRISPR/Cas9 in combination with a template DNA sequence can greatly increase the rate of HR. The HA length of donor template significantly changed the integration efficiency in BFF cells, which is consistent with previous studies (77, 80).

PFT-μ is a small molecule that inhibits p53 binding to mitochondria by reducing its affinity to the anti-apoptotic proteins Bcl-xL and Bcl-2 but has no effect on p53-dependent trans-activation (61). In this study, we found that addition of PFT-μ to the medium can significantly improve the efficiency of precise genome editing in BFF cells, which indicated that p53 may be one of the reasons for the decreased HDR efficiency in primary cells. PFT-μ can induce cell cycle arrest in acute leukemia cell lines (81), in addition HR is active only during the late S/G2 phase (82, 83). PFT-μ probably improve the knock-in efficiency of BFF cells by decreasing the apoptosis of cells with DSB, not by changing the cell cycle. Considering of the severe chromatin damage brought by p53 inhibition (84, 85), a short-term use of p53 inhibitors not only provide a window for genome editing temporarily, but also the expression level of p53 can restoration (59).

The selection of positive cells using drugs is a very crucial method for obtaining transgenic cells to produce transgenic animals (68, 86–88). Although the efficiency at which insertion/deletions are introduced by the CRISPR/Cas9 system is very high (54, 89), the knock-in efficiency still cannot satisfy the demands of agricultural production, which significantly limits the application of this technique (55). As has been indicated in many reports, drug selection can irreversibly harm the cells, the cells surviving drug screening had a decreased survival rate after multiple cell passages (55, 88).

DNA methylation and histone acetylation are important epigenetic events, the variations in the expression profile of epigenetic related genes in embryos and cloned animals are suggested to be linked with the reprogramming process (90–92). Some reports showed that the epigenetic modification of donor cells can affect the efficiency of reprogramming in SCNT (36, 37, 40). A previous research has shown that increased histone acetylation is associated with more effective formation of DNA replication complexes and can facilitate cell proliferation (93). In our study, no significant difference ($P < 0.05$) was observed in the transcript level of *DNMT1* and *DNMT3a*, and the expression level of *HDAC1*, *HDAC2*, *HDAC3*, which was found to be higher in Y-Chr-eGFP transgenic than in non-transgenic cells. The decreased histone acetylation levels may be one of the reasons for the lowered cell proliferative potential of transgenic cells and lowered cleavage rate of clone embryos from Y-Chr-eGFP transgenic BFF cells. Our results are consistent with Beyhan et al. who reported that the expression levels of HDACs in donor cells are related to the potential of embryos to develop to different embryonic stages (91). However, some research indicated the agent 5-aza-dC used for demethylation of DNA couldn't improve the development potential of cloned embryos (43, 44, 46), which indicated that the relationship between the level of DNA methylation status of donor cells and the developmental efficiency of cloned embryos needs to be further elucidated.

In conclusion, our study demonstrates that a Y chromosome tracer can be realized by CRISPR/Cas9 mediated HDR, p53 may be one of the reasons for the decreased HDR efficiency in primary cells. The raised expression level of *HDACs* in Y-Chr-eGFP transgenic BFFs could be correlated with reduced cell proliferative potential and developmental potential of transgenic cloned embryos. As far as we know, this is the first report of applying CRISPR/Cas9-mediated gene editing to solve the challenge of controlling the sex ratio of domestic animals. This work provides a rapid and non-invasive method to identify the sex of mammalian implantation embryos and lays a solid

foundation for the generation of Y-Chr-eGFP transgenic buffalo and other transgenic species.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care & Welfare Committee of Guangxi University (GXU2016-013).

AUTHOR CONTRIBUTIONS

MZ, XZ, and JN conceived the study. XZ, JN, YT, WH, KX, and CP performed the experiments. XZ, JN, XL, YL, and MZ analyzed the data and wrote the manuscript. All authors reviewed the manuscript and approved the final version of the paper.

FUNDING

This work was supported by the open program of State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources (No. SKLCUSA-b201914), the open program of key Laboratory of Buffalo Genetics, Breeding and Reproduction Technology of Guangxi Province (No. SNKF-2019-01).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00199/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 14 May 2020
doi: 10.3389/fvets.2020.00269

Identification of Pathogenic *Leptospira* Species in the Urogenital Tract of Water Buffaloes (*Bubalus bubalis*) From the Amazon River Delta Region, Brazil

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OPEN ACCESS

Edited by:
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Institut Pasteur de Nouvelle Calédonie, New Caledonia

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 28 November 2019
Accepted: 21 April 2020
Published: 14 May 2020

Citation:
Guedes IB, Oliveira de Souza G, Fernandes de Paula Castro J, de Souza Filho AF, Cavalini MB, Taniwaki SA, Maia ALP, Pereira IC and Heinemann MB (2020) Identification of Pathogenic Leptospira Species in the Urogenital Tract of Water Buffaloes (Bubalus bubalis) From the Amazon River Delta Region, Brazil. Front. Vet. Sci. 7:269. doi: 10.3389/fvets.2020.00269

In the current context of deforestation and fire in the Amazon, buffaloes could be a cost-effective and sustainable alternative for cattle production in the region, as they can convert low-quality foods and be raised in floodplain areas. However, little is known about the reproductive diseases that affect these animals; thus, the purpose of this study was to perform the molecular characterization of *Leptospira* spp. in the urogenital tract of water buffaloes (*Bubalus bubalis*) raised in the Amazon River Delta region in Brazil. Samples were collected from 114 kidneys, 204 ovaries, and 160 uterine swabs of slaughtered buffaloes in the Macapá microregion of Amapá State (Brazil) and were subjected to PCR to detect bacterial DNA. Positive amplicons were sequenced to identify *Leptospira* species. Among the total samples, 11/473 were PCR positive (2.3%), including 10 kidney samples and one uterine swab sample. DNA sequencing identified two pathogenic species from the kidney samples: *L. interrogans*, accounting for 60.0% (6/10) of these samples, and *L. borgpetersenii*, accounting for 20.0% (2/10), while 20.0% (2/10) were identified only at the genus level. The bacterium in the uterine swab sample was identified as *L. interrogans* with genetic proximity to strains belonging to the serovar Hardjo. This is the first report of leptospires species identified in buffaloes from the Amazon River Delta region and revealed that these animals may be carriers of different pathogenic *Leptospira* species, similar to bovines, including showing genital colonization.

Keywords: ***Leptospira*, buffalo, PCR, DNA sequencing, Amazon**

INTRODUCTION

Leptospirosis is a bacterial disease that affects humans and several species of domestic and wild animals and is considered a zoonosis (1). The genus *Leptospira* can be considered to be dynamic and diversified in relation to the species it includes; with the advancement of molecular methodologies, it has been possible to define 64 species, which are now divided into two major clades, one of which contains pathogenic species, while the other contains saprophytes, leading to a new proposal for

the systematic classification of the genus (2). Nevertheless, the serological classification of leptospires is still accepted and is the basis of serological assays such as the microscopic agglutination test (MAT), in which a limited number of serovars that represent prevalent serogroups for a specific region are used (1, 3).

Similar to the disease in cattle, buffalo leptospirosis is mainly characterized by reproductive disorders, especially abortions, in which *Leptospira* spp. have been detected (4, 5). In Brazil, there has been only one report of *Leptospira* isolation from the urine of a healthy buffalo from the southeastern region of the country, and the isolate was classified as *L. santarosai* serovar Guaricura (6). Due to the difficulties in isolating leptospires from biological samples, direct DNA sequencing from PCR products is used, which allows the identification of leptospires at the species level, providing a new epidemiological analysis of the disease (7–9).

In 2018, the Brazilian buffalo herd consisted of 1,390,066 buffaloes, and ∼37% of this herd was concentrated in the Amazon River Delta region (considering the east coast of Amapá state and Marajó Island) (10). Buffaloes are part of the Amazonian culture since it is used as a work animal and it plays a role in tourism, so these animals are in close proximity to humans (11). This close contact may represent a public health problem since leptospirosis is a zoonosis, and it has been reported that buffaloes can be directly involved in the transmission of leptospirosis to humans (12). In this region, serological studies have revealed the presence of anti-*Leptospira* spp. antibodies in buffaloes, with a prevalence ranging from 34.37 to 80.0% and predominance of the Sejroe serogroup (13–15), similar to what is observed in cattle. Nevertheless, there have been few studies on leptospirosis in buffaloes; thus, the aim of this study was to detect and perform the molecular characterization of *Leptospira* spp. in the urogenital tract of buffaloes raised in the Amazon River Delta region, providing new knowledge about leptospirosis in these animals.

MATERIALS AND METHODS

This work was approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science (Universidade de São Paulo)—CEUA/FMVZ n° 5613211118. For this study, 114 kidney fragments (∼5 g), 204 ovaries and 160 uterine swabs were collected from buffaloes slaughtered in a slaughterhouse in the Macapá microregion of Amapá State, Brazil. Sampling was carried out by convenience, and for logistical reasons during slaughter, the samples were collected in groups on different days according to the type of tissue (kidneys—day 1, ovaries—day 2, uterine swabs—day 3); thus, each sample represented a single animal, totaling 478 animals.

The animals came from different farms located in the Amazon River Delta region in northern Brazil, which is characterized as a geographical area formed by hundreds of islands and islets between the states of Pará and Amapá, encompassing Marajó Island (**Figure 1**). The animals predominantly belonged to the River Buffalo group breeds (Murrah, Mediterranean, and Jafarabadi) and included males and females that were at least 12 months old, showed meat production capacity, had not been vaccinated against leptospirosis and had an unknown reproductive history.

During the slaughter of the animals, fragments of the kidneys, and ovaries were collected with the aid of sterile forceps and scissors and stored individually in sterile plastic bags for homogenization. At the slaughterhouse immediately after collection, the organs were macerated and diluted 1:10 in phosphate-buffered saline [PBS; 0.137 M NaCl; 0.0027 M KCl; 0.01 M Na_2HPO_4; and 0.0018 M KH_2PO_4 (pH 7.4)]. The uterine swab samples were obtained with the aid of a disposable cervical brush fixed to a swab, which was vigorously rubbed against the mucosa of the uterine horns and body, and were also diluted in phosphate-buffered saline. A 1 ml aliquot of the diluted samples was frozen and sent to the laboratory for tests.

The extraction and purification of DNA from the samples were performed using the PureLink® Genomic DNA Mini Kit (Invitrogen™) according to the manufacturer's protocol. PCR targeting the *Bubalus bubalis* cytochrome b (*cytb*) gene, as described by Bottero et al. (16), was used as an internal control to verify the validity of the extracted DNA. The detection of *Leptospira* spp. was carried out by PCR with the Lep1 and Lep2 primer pair, which amplifies a 330 bp region of the 16S rRNA gene (*rrs*) (17), using Go Taq™ Green Master Mix (Promega, Brazil). The positive samples were subjected to another round of PCR for typing using primer pairs that amplify a 549 bp region of the *secY* gene (18). *L. interrogans* serovar Hardjo-prajitno and ultrapure water were used as positive and negative controls, respectively. A negative control sample was inserted between every five test samples to assess the presence of contamination in the extracted DNA.

Positive amplicons were sequenced by the Sanger method with BigDye Terminator v.3.1 chemistry (Applied Biosystems) and an ABI-3500 (Applied Biosystems) automatic sequencer according to the manufacturer's instructions. The sequences were assembled with the BioEdit Sequence Alignment Editor (19). The phylogenetic trees were built using homologous sequences retrieved from the GenBank database (accession numbers in **Figures 2**, **3**) with the neighbor-joining method, the Tamura-3-parameter model and 1,000 bootstrap replicates in MEGA 7 software (20).

RESULTS

Among the total samples (478) subjected to the internal control PCR assay, only five ovarian samples were not amplified and were disregarded, resulting in a total of 473 samples that were analyzed. In the *rrs* PCR analysis, 11/473 samples were positive (2.3%), 10 of which were kidney samples, and one was a uterine swab. All ovarian samples were negative. None of these positive kidney samples showed amplification of the *secY* gene. However, the *rrs*-positive amplicons were sequenced (GenBank accession numbers from MN720219 to MN720228), and it was possible to identify two pathogenic species: *L. interrogans*, accounting for 60.0% (6/10) of these samples, and *L. borgpetersenii*, accounting for 20.0% (2/10), while 20.0% (2/10) of the samples were identified only to the genus level and also corresponded to pathogenic species (**Figure 2**).

FIGURE 1 | Amazon River Delta region in Brazil (circle).

The positive uterine swab sample was identified as *L. interrogans* by the partial sequencing of the *secY* gene (GenBank accession number: MN723893) and showed genetic proximity to strains belonging to the serovar Hardjo (**Figure 3**). As each sample represented a single animal, it was not possible to correlate the samples with each other.

DISCUSSION

Although buffaloes are considered rustic, as reflected in their natural resistance to disease in the environment, these animals can be affected by infectious agents, including those related to reproductive disorders (21). In this work, the identification of bacterial DNA in animals showed that buffaloes may be as sensitive to *Leptospira* spp. as cattle, including showing renal colonization and, consequently, the elimination of bacteria through the urine, which becomes a source of environmental contamination (22).

In the Amazon, climatic conditions may favor the occurrence of leptospirosis in cattle and buffalo, since the risk of animals contracting the disease is higher in regions affected by seasonal flooding (23). In bovines, the control of leptospirosis is difficult because different strains of leptospires may be adaptable to cattle (24) and the disease is not yet well-understood in buffalo, as few studies have provided information about the species of *Leptospira* circulating in these animals.

The direct sequencing of PCR products from kidney samples showed that the pathogenic species *L. interrogans* was most prevalent (6/10) in these samples, whereas the *L. borgpetersenii* is usually reported to be the most prevalent species in cattle (7, 9). In other animal species such as ewes and horses, *L. interrogans* is most commonly found in the genital tract (25, 26); in association with such infections, recurrent uveitis, a classic leptospirosis injury in horses, seems to be directly related to strains of *L. interrogans* (27). Two sequences (MN720221 and MN720222) formed two independent clades and could not be identified at the species level, but is suggested that this two samples are *L. borgpetersenii*-like species, as these two samples showed >98.9% identity with *L. borgpetersenii*; in particular the MN720222 sample was homologous to *L. borgpetersenii* isolated from a human patient in Guayaquil, Ecuador (28).

A difficulty found in the present study was that the kidney samples positive for the *rrs* gene could not be amplified by PCR targeting the *secY* gene. This situation has also been observed in other studies, and the discrepancy between PCR assays could arise due to the amount of original bacterial DNA in the material as well as the quality of the sample tested (7, 9). In addition, not all PCR products can always be sequenced (29) or generate interpretable data (30).

Despite the limitations of the use of the *rrs* gene to separate *Leptospira* species (2), this target and *secY* gene, which are used for the molecular characterization of leptospires, have many

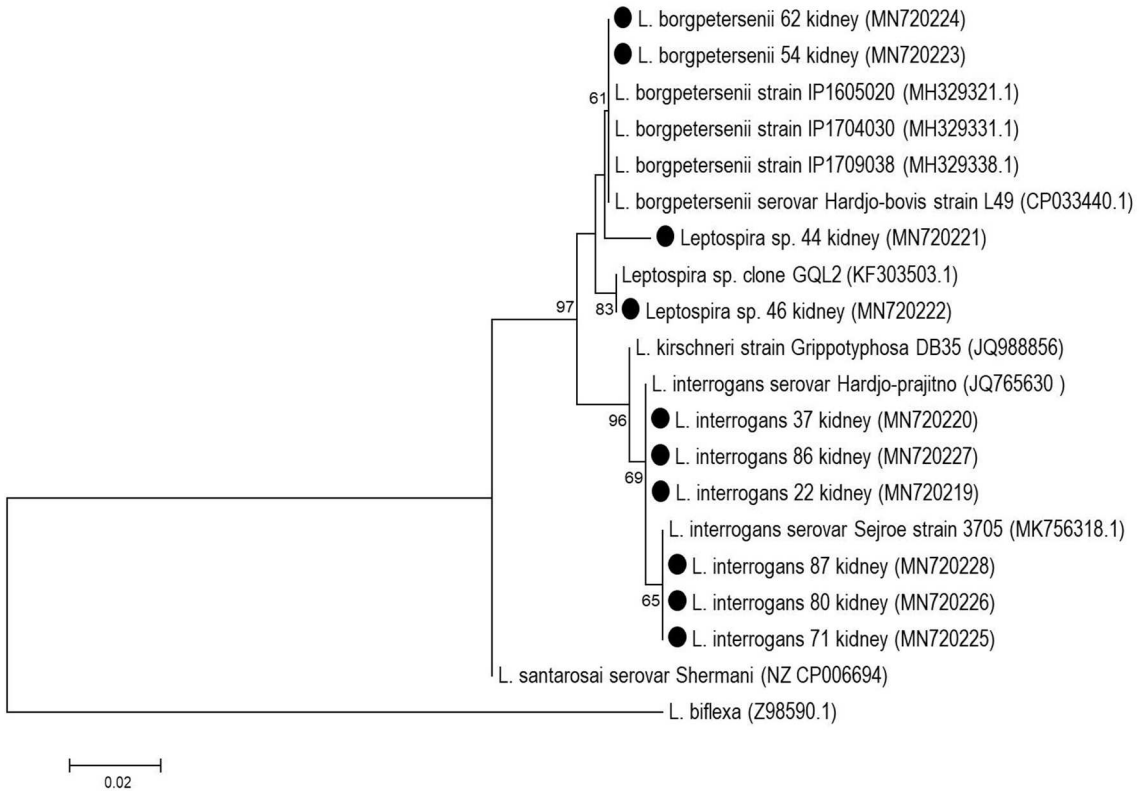

FIGURE 2 | Phylogenetic reconstruction based on partial *rrs* gene of *Leptospira* spp. from kidney samples of buffaloes (black circles). The tree was elaborated with Neighbor-Joining method, Tamura-3 parameter model with bootstrap test of 1,000 replicates.

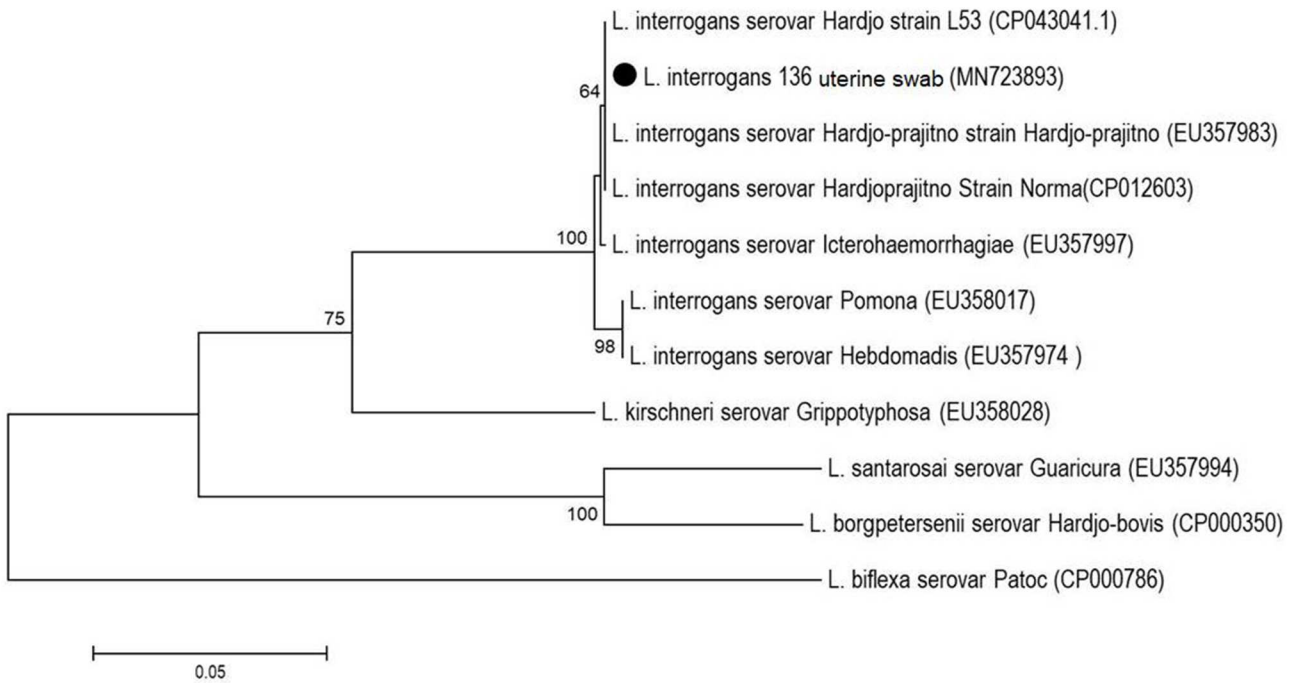

FIGURE 3 | Phylogenetic analysis based on partial *secY* gene of *Leptospira* spp. from the uterine swab sample (black circle). The tree was elaborated with Neighbor-Joining method and Tamura-3 parameter model with bootstrap test of 1,000 replicates.

characterized samples deposited on GenBank which facilitates sample typing (8, 31–33). Increasingly, different target genes have been described and proposed to assist in the molecular characterization of leptospires (*lipL32*, *flaB*, *gyrB*, *rpoB*, among others) which had success both in characterizing isolates and directly from PCR products, although this last option is less sensitive. These targets can generate "barcodes" which have variable discriminatory power, and can be combined to increase the differentiation of *Leptospira* species (34).

An interesting result was that *L. santarosai* was not detected in the buffalo samples, differing from what is reported for cattle in other regions of the country, such as the southeast, where this species is one of the most prevalent (8, 24, 35). The dimensions and heterogeneity of the with climatic conditions in the Amazon make this region unique and different from other parts of the country, which is reflected in the etiological agent-host relationships and may account for the situation found in cattle from the Amazon region, where *L. santarosai* may not be involved in leptospirosis in these animals (9, 36). Regarding buffalo, more work is needed to evaluate the participation of *L. santarosai* and others species in buffalo leptospirosis.

No ovarian specimens were PCR positive, suggesting that this organ is not be a preferred site for the colonization of leptospires in buffalos, although there has been one report of leptospiral DNA detected in the ovaries of slaughtered sheep (37). The isolation of *L. interrogans* from ovaries has been reported, but only in experimentally infected hamsters in the acute phase of the disease (38). The potential adaptability of leptospires to their hosts, especially in cattle, leads to a chronic character of these infections, where extrarenal colonization by leptospires has been described focusing on the structures of the female reproductive system, such as the vagina, uteri, and oviducts (39–41).

The positive uterine swab sample (MN723893), belonging to *L. interrogans*, showed homology to sequences characterized as belonging to serovar Hardjo strains, including an autochthonous sample (CP043041.1) isolated from the urine of dairy cattle in Paraná, in the southern region of Brazil (42). This finding may suggest that sexual transmission may be occurring among these animals. The presence of *Leptospira* in the reproductive tract of animals (25, 40, 41) demonstrates the tissue tropism of the bacterium, causing reproductive disorders, especially the strains of the Sejroe serogroup, which are considered to be adapted to cattle, promoting chronic infection of the genital tract and subtly compromising the reproductive performance of these animals for long periods. This condition has recently been described as a syndrome, referred to as bovine genital leptospirosis (BGL) (43).

CONCLUSION

This is the first report of leptospires species identified in buffaloes from the Brazilian Amazon and revealed that these animals may be carriers of different pathogenic *Leptospira* species, similar to bovines. Genital colonization was found, showing that the reproductive system of the buffaloes may be affected.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Genbank NCBI. Accession numbers from MN720219 to MN720228 and Genbank accession number: MN723893.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo).

AUTHOR CONTRIBUTIONS

IG, AM, and IP carried out the collection of samples at the slaughterhouse. IG, GO, JF, AF, and MC performed all the laboratory tests. IG and ST performed the interpretation of DNA sequencing results. IG wrote the manuscript and ST did the translation. MH accurately reviewed the manuscript. All authors have read and approved the final version of the manuscript.

FUNDING

This study was financed in part by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), Brasil – Finance Code 001 and by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico)–(420110/2018-6).

ACKNOWLEDGMENTS

MH thanked CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) the fellowship. IG thanked CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the scholarship.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 19 May 2020
doi: 10.3389/fvets.2020.00180

Infrared Thermography to Assess Thermoregulatory Reactions of Female Buffaloes in a Humid Tropical Environment

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OPEN ACCESS

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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: 15 January 2020
Accepted: 19 March 2020
Published: 19 May 2020

Citation:
Brcko CC, Silva JAR, Martorano LG, Vilela RA, Nahúm BS, Silva AGM, Barbosa AVC, Bezerra AS and Lourenço Júnior JB (2020) Infrared Thermography to Assess Thermoregulatory Reactions of Female Buffaloes in a Humid Tropical Environment. Front. Vet. Sci. 7:180. doi: 10.3389/fvets.2020.00180

This study employed infrared thermography to assess the thermoregulatory responses of female buffaloes reared in a hot and humid climate as a function of variations in time and the thermal environment, and to correlate rectal temperature with that of different body areas. The trial was carried out at Embrapa Eastern Amazon (01°26'S and 48°24'W) in Belém, PA, Brazil. Twenty-four female buffaloes fed exclusively on pasture and exposed to the sun throughout the day in an open area were used. The animals were taken back to the corral at 6 A.M., 9 A.M., 12 P.M., 3 P.M., 6 P.M., and 9 P.M. for measurements of rectal temperature (RT), respiratory rate (RR), and body surface temperature (BST) using infrared thermography (IRT). Data on air temperature (AT), relative air humidity (RH), wind velocity (WV), and solar radiation (SR) were also recorded. A quadratic effect of AT, RH, SR, RR, and RT ($P < 0.01$) was found as a function of measurement time. Between 12 P.M. and 3 P.M., AT, SR, RR, and RT values were the highest, while RH values were the lowest ($P < 0.05$). WV was the highest between 12 P.M. and 9 P.M. A difference was found in RR and RT among the day's times, with the highest values at 12 P.M. and 3 P.M. ($P<0.05$). The IRTs have a quadratic effect as a function of day's times, with the highest levels between 12 P.M. and 6 P.M. and a reduction at 9 P.M. All IRTs are correlated with the physiological variables RR and RT, except for the IRT of the side of the back, which is not correlated with RT. The IRTs of the eye and cheek are correlated with RT ($P < 0.01$) and with RR ($P < 0.05$). IRT was efficient in evaluating the thermal stress of female buffaloes in hot and humid climates, and the technique can be used to evaluate the body temperature of these animals, since the RT was positively correlated with the temperature of the eye and cheek.

Keywords: buffaloes, climate, heat, images, thermoregulation

INTRODUCTION

Buffaloes (*Bubalus bubalis*) were introduced in Brazil in 1895, in the Island of Marajó, PA (1), and the current herd is estimated at 1.4 million heads. The country's North region accounts for most of that with 922,600 heads, or 66.4% of the national herd, followed by the Southeast and Northeast regions, with 188,100 and 125,300 thousand heads, respectively. Currently, buffalo farming in this region provides meat, milk, leather, and horns and is concentrated mainly on small farms (2).

Buffaloes are known for their rusticity in adverse environments and represent an option to make use of areas in a ranch to which bovines do not adapt (3). However, the difficulty these animals have in releasing body heat under high temperature and humidity may negatively impact their productive and reproductive performances (4). Buffaloes in hot conditions have increased blood flow on their skin surfaces so as to raise its temperature and facilitate heat loss through conduction when the animals are immersed in mud or water (5).

The use of infrared thermography (IRT) poses an alternative to assess the body surface temperature (BST) since it enables obtaining the heat irradiating in the infrared spectrum without interfering on animals' behavior (6). This technique is innovative as it can be used with no direct interaction with the animal, thus being considered a non-invasive tool.

In face of that, the present research aimed to assess the thermoregulatory responses of female buffaloes reared in the hot and humid climate of the Eastern Amazon as a function of variations in time and thermal environment, besides employing thermography to map the BST of buffaloes in order to find the area that is most associated with rectal temperature (RT), hence contributing to animal biometeorology studies.

MATERIALS AND METHODS

The experiment was approved by the Ethics Committee on Experimental Animal Research of the Federal University of Pará under the protocol BIO 120-13. The experimental sample consisted of 24 non-pregnant, non-lactating, clinically healthy female crossbred Murrah/Mediterranean buffaloes (*Bubalus bubalis bubalis*) whose initial mean age and weight were 54 ± 7 months and 503.1 ± 23 kg, respectively. The animals were selected according to their body score aiming at greater uniformity and were kept in 1-ha pens with *Brachiaria brizantha* (cv. Marandu) grass in a rotational system with no access to shade or ponds and with drinking water and mineral salt *ad libitum*.

The trial lasted for six consecutive days. The buffaloes were weighed before and after the trial. Such information was used to calculate heat storage and later to calculate the accumulated heat following the recommendations by McGovern and Bruce (7).

Heat storage calculation, in kelvin kg^{-1} h^{-1}:

$$\boldsymbol{\Delta RT} = \frac{(\boldsymbol{3{,}600.Harm.A})}{(\boldsymbol{Mv.Hb})} \quad \text{where :}$$

ΔRT—difference in RT;
H_{arm}–stored heat ($W.m^{-2}$);
A—animal's surface (m^2) calculated by the equation: $A=0.13.M_v^{0.556}$;
M_v–body mass (kg);
H_b–animal's specific heat (3,400 kJ kg^{-1} K^{-1}).

About 30 min prior to collections, animals were taken to the corral about 300 m away from the pen, where they were contained at 6 A.M., 9 A.M., 12 P.M., 3 P.M., 6 P.M., and 9 P.M. for the measurement of the physiological variables of RT, respiratory rate (RR), and BST measured through IRT, with ± 1°C accuracy and a spectral range of 7.5–13.0 μm.

A clinical veterinary thermometer graded in Celsius, with decimal accuracy, was inserted 5 cm into animals' rectum for 1 min to measure the RT. RR was obtained through inspection and by counting the thoracic-abdominal movements for 1 min with the aid of a stopwatch. The animals randomly entered the holding pens.

The IRTs were obtained using a FLIR T E30 thermographic camera with automatic calibration. The measurements were determined by the average readings of two areas delimited in the image by markers. All images were acquired from the animals' right side so as to reflect the actual IRT fluctuation, thus preventing digestive processes in the rumen from impacting the increase in BST. Each thermogram was saved to a memory card and later analyzed in the software FLIR Tools, where the mean temperatures of each region were obtained considering emissivity of 0.98. Images were taken from five areas, namely, side of the back, tail insertion, eye, cheek, and hind ergot (**Figure 1**). Approximately 2,592 thermographic images were acquired during collection days. The ones that lacked the clarity required for the analysis in the software were excluded.

The distance used to take the photographs (distance between the camera and the evaluated area) was 30 cm for eye, cheek, and hind ergot and 1 m for side of the back and tail insertion.

The meteorological data of air temperature (AT,°C) and relative air humidity (RH, %) were recorded using a U12-012 HOBO® data logger (Onset, Brazil) installed at the experimental site. A TAD-800 portable digital thermo-anemometer (Instrutherm, São Paulo, Brazil) was used to measure wind velocity (WV, m/s). Solar radiation (SR, $MJ.m^{-2}$) was obtained from INMET's (National Institute of Meteorology) station 3.5 km away from the experimental site. The environmental variables were read along the day at the same time as the physiological variables.

The statistical analysis of data was performed in the software Statistical Analysis System (SAS 9.1). The physiological responses (RT, RR, BST, and IRT) and climate variables (AT, RH, WV, and SR) were analyzed using general linear models (GLMs) with two statistical models. In the first one, for physiological variables, repeated measures are made on the same experimental unit (animal) over a period of time. The model is represented by: $Y_{ijk} = \mu + a_i + b_{icp} + e_{ijk}, y_{ijk} = \mu + \alpha_i + b_{i(j)} + e_{ijk}$ in which Y_{ijk} refers to the value of the ith animal obtained at the jth hour of the kth day; $\mu\mu$ is the overall mean; a_i is the fixed effect of the jth hour of the day; b_{icp} is the random effect of the ith animal belonging to the jth hour of the day; and e_{ijk} refers to the residual including the random error. The second statistical model, for climate variables, is: $Y_{jk} = \mu + aj + e_{jk,}$ in which Y_{jk} refers to the value obtained

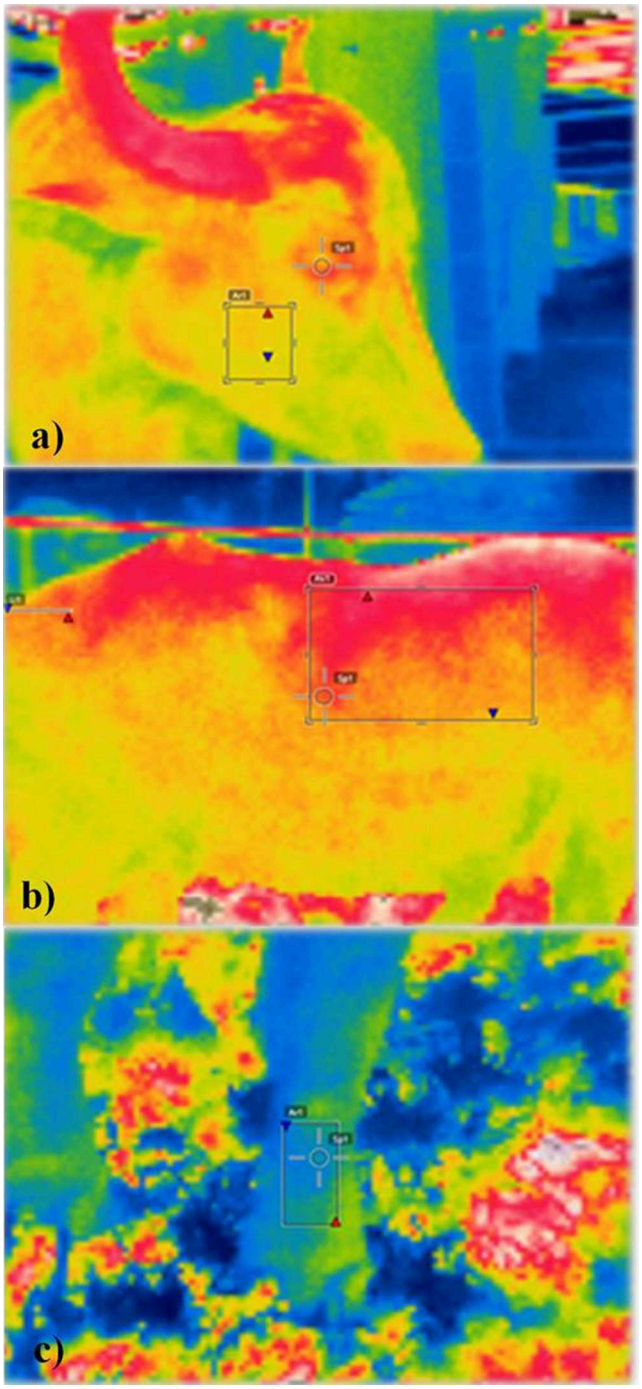

FIGURE 1 | (a) Cheek, eye, **(b)** side of the back, tail insertion point, and **(c)** ergot of female buffaloes reared in the Eastern Amazon.

at the jth hour of the kth day; μ is the overall mean; aj is the fixed effect of the jth hour of the day; and e_{jk} refers to the residual including the random error.

To determine the linear relationship between BST [eye temperature (°C), cheek temperature (°C), side-of-back temperature (°C), tail temperature (°C), and ergot temperature (°C)] and RR and RT, Pearson correlation was calculated by the procedure PROC CORR in SAS. To perform regression in SAS between RR and time of the day and RT and time of the day, the procedure PROC REG (means adjusted by least square) was employed by a quadratic polynomial model: $Y = \beta_0 + \beta_1 X + \beta_2 X^2$, $Y = \beta_0 + \beta_1 X + \beta_2 X^2$in which Y is the physiological variable [eye temperature (°C), cheek temperature (°C), side-of-back temperature (°C), tail temperature (°C), ergot temperature (°C), RR (mov/min), and RT (°C)] and X is the time of the day (6 A.M., 9 A.M., 12 P.M., 3 P.M., and 9 P.M.).

TABLE 1 | Number of observations (N), mean, standard deviation (SD), coefficient of variation (CV), minimum (Min), and maximum (Max) values of agrometeorological variables and RR and RT of female buffaloes during the experimental period in the Eastern Amazon.

Variable	N	Mean	SD	CV (%)	Min	Max
AT	858	29.26	3.74	12.78	23.14	37.28
RH	858	74.50	10.97	14.72	47.62	90.06
THI	858	80.56	4.21	5.22	72.45	87.26
SR	858	968.34	1,013.52	102.76	−3.54	2,844
WV	858	0.97	0.81	83.50	0	2.5
RR	858	27.5	15.4	56.00	11	103
RT	858	38.8	0.48	1.24	30	40.7

AT, air temperature (°C); RH, relative humidity (%); THI, temperature and humidity index; SR, solar radiation (kJ m^{-2}); WV, wind velocity (m s^{-1}).

RESULTS

An interaction can be seen among the meteorological variables and the day's times. The mean, maximum, and minimum values, the number of observations (N), standard deviation (SD), and coefficient of variation (CV) of climate variables and RR and RT of female buffaloes are presented in **Table 1**.

The regression analysis associated with AT as a function of measurement time showed a quadratic model ($P < 0.01$). By deriving the equation, the highest AT value of 32.9°C was found at 1:42 P.M. It was observed that RH's effect is the opposite of AT's, with the lowest value of 64% at 1:27 P.M. ($P < 0.05$).

A quadratic effect was found as a function of assessment times in the regression associated with SR ($P < 0.01$). The maximum radiation value, by deriving the equation, was 1,639 MJ m^{-2} at 1:35 P.M.

The regression analysis regarding RR showed a quadratic effect ($P < 0.01$) as a function of the time of day (**Figure 2**). By deriving the equation, the time of 1:28 P.M. was found for maximum RR, which would reach 36.08 mov min^{-1}. A gradual increase in RR was observed up until 1 P.M., starting at 18.69 mov min^{-1} at 6 A.M., climbing to 27.08 mov min^{-1} at 9 A.M., and reaching mean values of 41.04 mov min^{-1} at 12 P.M.

A significant difference ($P < 0.05$) was found in RT along the day, with the highest value (39.01°C) at 3 P.M. The regression coefficient analysis associated with RT differed ($P < 0.01$) among the assessment times following a quadratic model, as seen in **Figure 3**. By deriving the equation, the time of 3:05 P.M. was obtained for maximum RT, which would reach 38.63°C. The thermal balance dynamics of female buffaloes observed at 6 A.M., 9 A.M., 12 P.M., 3 P.M., 6 P.M., and 9 P.M. during

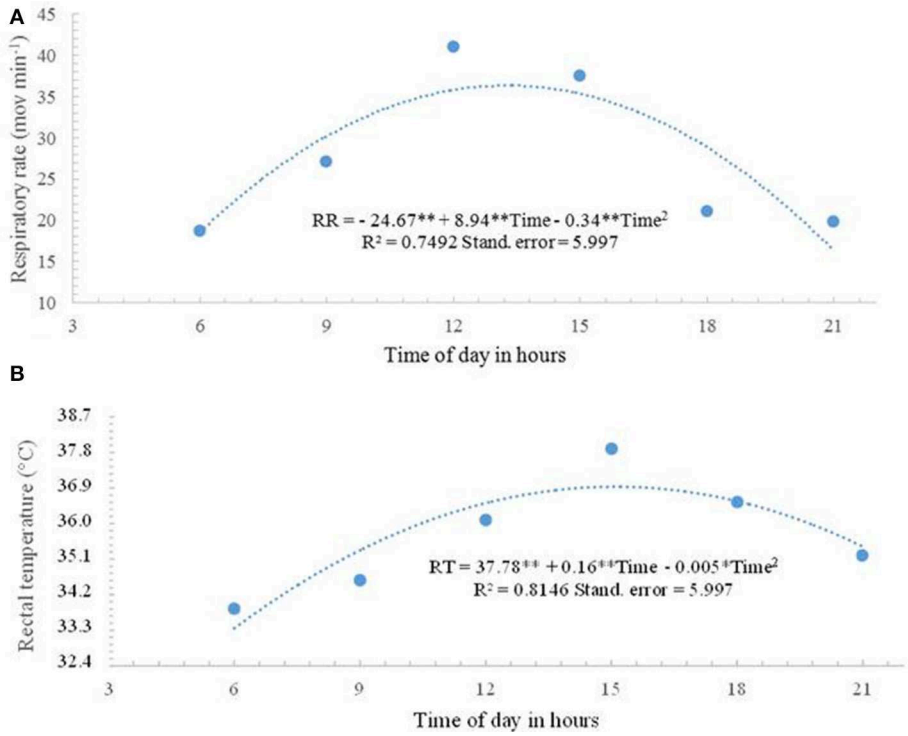

FIGURE 2 | (A) Respiratory rate (RR) and **(B)** rectal temperature (RT) of female buffaloes observed at 6 A.M., 9 A.M., 12 P.M., 3 P.M., 6 P.M., and 9 P.M. during the experimental period in the Eastern Amazon. $^{*}p < 0.05$ $^{**}p < 0.01$.

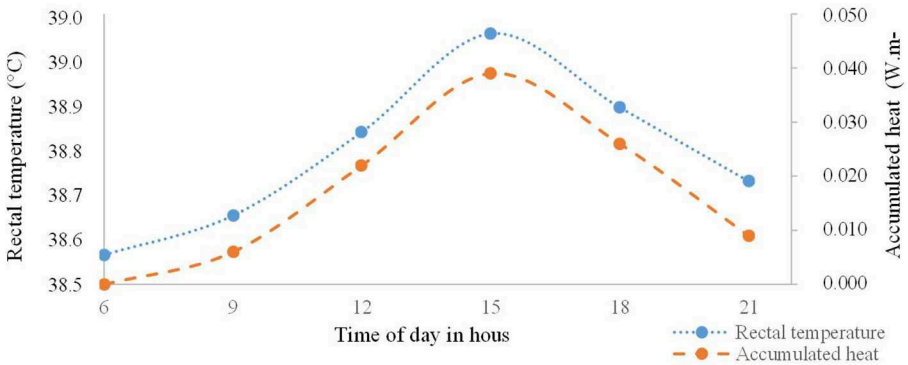

FIGURE 3 | Thermal balance dynamics of female buffaloes observed at 6 A.M., 9 A.M., 12 P.M., 3 P.M., 6 P.M., and 9 P.M. during the experimental period in the Eastern Amazon.

the experimental period in the Eastern Amazon is presented in **Figure 4**.

The regression analysis of IRTs showed a quadratic effect as a function of the times of the day. By deriving the equations, the following times would yield maximum IRT results for different parts of the body: 1:50 P.M. for the eye at 36.49°C (**Figure 4A**); 1:51 P.M. for the cheek at 36.6°C (**Figure 4B**); 1:51 P.M. for the back at 38.71°C (**Figure 4C**); 2:23 P.M. for the ergot at 37.01°C (**Figure 4D**); and 1:54 P.M. for the tail at 33.81°C (**Figure 4E**).

The correlation among IRTs and physiological variables is in **Table 2**. All IRTs are correlated with the RR and RT, except the IRT of the side of the back, which is not correlated with RT, although it is correlated ($P < 0.01$) with RR. The IRTs of the eye and cheek are correlated with RT ($P < 0.01$) and RR ($P < 0.05$). Likewise, the IRTs of the tail and ergot are correlated ($P < 0.05$) with RR and RT.

DISCUSSION

The highest AT value of 32.9°C was found at 1:42 P.M., which shows that the hotter climate at that time could be detrimental to the animals' thermal comfort. Under high AT

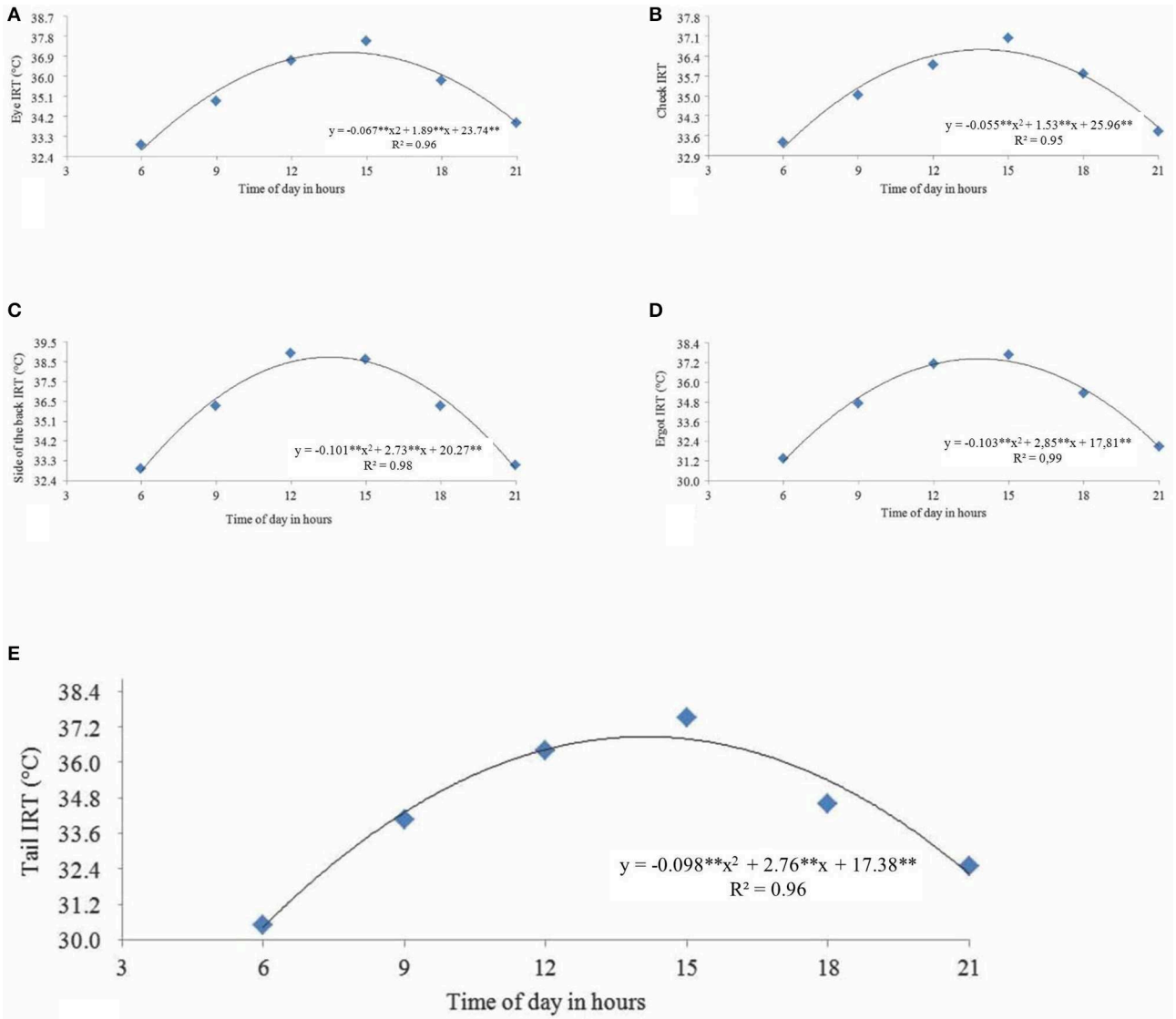

FIGURE 4 | (A) Eye, **(B)** cheek, **(C)** side of the back, **(D)** ergot, and **(E)** tail infrared thermography (IRT) of female buffaloes observed at 6 A.M., 9 A.M., 12 P.M., 3 P.M., 6 P.M., and 9 P.M. during the experimental period in the Eastern Amazon. $^{**}p < 0.01$.

and RH, animals dissipate heat and their physiological variable values change (8). It was observed that RH's effect is the opposite of AT's, with the lowest value of 64% at 1:27 P.M. ($P < 0.05$). Similar AT and RH values were reported by Silva et al. (9).

SR directly impacts the animals reared in a traditional system with no access to shade. Buffaloes are particularly susceptible to direct SR since they absorb a lot of heat because of their dark skin and sparse fur (10). The direct incidence of radiation onto the animals contributes to the increase in BST and further raises AT. Studies conducted in the Amazon region confirm that this environment is conducive to causing thermal stress in buffaloes, especially in the afternoon, and that the use of shading, as in a silvipastoral system, would facilitate body heat dissipation (11, 12).

TABLE 2 | Correlations among body surface temperatures assessed by infrared thermography and physiological variables of respiratory rate and rectal temperature of female buffaloes reared in a hot and humid climate.

Body surface temperature	Respiratory rate	Rectal temperature
Eye temperature (°C)	0.85303*	0.92904**
Cheek temperature (°C)	0.81573*	0.92226**
Side of back temperature (°C)	0.92965**	0.78412^{NS}
Tail temperature (°C)	0.88633*	0.86125*
Ergot temperature (°C)	0.87408*	0.89005*

*$P < 0.05$; **$P < 0.01$, ^{NS}, Nonsignificant.*

The maximum radiation value by deriving the equation was 1,639 MJ m^{-2} at 1:35 P.M. Along the day, some moments are more or less favorable to animals' thermal comfort, and this

process is mediated by the radiation balance, i.e., the difference between the radiation received and given back, which greatly varies along the day and the year and promotes daily and annual changes in AT (13).

The higher AT leads to a reduction in the thermal gradient between the animal and the environment, which hinders the sensible dissipation of accumulated metabolic heat (14). In this scenario, evaporative thermolysis becomes an important pathway to maintain this thermal balance. RR is an important indicator of animal thermal stress in hot environments because, in this situation, the animal's respiratory frequency increases to eliminate body heat through evaporative heat loss (15).

By deriving the equation, the time of 1:28 P.M. was found for maximum RR, which would reach 36.08 mov min^{-1}. The higher RR values between 12 P.M. and 3 P.M. are associated with the increase in AT and SR. RR is relevant in the dissipation of endogenous heat in buffaloes.

According to Alam et al. (16), the thermal stimulation of peripheral thermoreceptors sets off evaporative thermolysis through polypnea. According to that reasoning, the animals accumulated heat along the day from SR, which led to higher RT despite them losing heat through the convection and latent pathways. After RT decreased, thermal balance was reestablished because the animals more effectively used sensible and latent thermolysis.

In the present research, a gradual increase in RR was observed up until 1 P.M., starting at 18.69 mov min^{-1} at 6 A.M., climbing to 27.08 mov min^{-1} at 9 A.M., and reaching mean values of 41.04 mov min^{-1} at 12 P.M. Similar values were reported by Silva et al. (17). The highest value of RT (39.01°C) was observed at 3 P.M. By deriving the equation, the time of 3:05 P.M. was obtained for maximum RT, which would reach 38.63°C.

It is seen that, even when AT was milder, RT values were beyond the normal range for buffaloes, i.e., 37.4 to 37.9°C (18). Since they have very pigmented skin and low fur density, when buffaloes are exposed to SR, the radiant energy is absorbed and transmitted following a centripetal heat flow (3). At 3 P.M., with SR of 2,443.1 MJ m^{-2}, the animals' RT was very high (39.01°C), which suggests that the heat release mechanisms had become insufficient to maintain homeothermy. Later, as AT decreased, a change in heat flow following a centrifugal path was observed, with greater thermal inertia for heat dissipation. RT decreased between 6 P.M. and 9 P.M. to the point of reestablishing thermal balance.

Along the day, heat gain through radiation influenced the heat accumulated by the animals and, consequently, led to higher RT. The moment RT decreased, animals were able to regain thermal balance.

The following times would yield maximum IRT results for different parts of the body: 1:50 P.M. for the eye at 36.49°C; 1:51 P.M. for the cheek at 36.6°C; 1:51 P.M. for the back at 38.71°C; 2:23 P.M. for the ergot at 37.01°C; and 1:54 P.M. for the tail at 33.81°C. These results demonstrate that the hottest times of the day influence these variables.

The correlation among the IRTs and the physiological variables RR and RT may show the efficacy of these variables in indicating thermal stress. All IRTs are correlated with the RR and RT, except the IRT of the side of the back, which is not correlated with RT, although it is correlated ($P < 0.01$) with RR. The IRTs of the eye and cheek are correlated with RT ($P < 0.01$) and RR ($P < 0.05$). Likewise, the IRTs of the tail and ergot are correlated ($P < 0.05$) with RR and RT. These results show that the IRTs of the eye and cheek are the most appropriate to assess thermal stress in buffaloes since they have the best correlations with RT, which indicates thermal balance and is used to assess the adversity of the thermal environment (19).

Researches carried out on buffalo bulls (20) and dairy buffalos (21) also concluded that the IRT technique was efficient to identify the thermal stress in these animals raised in tropical climate.

Using the IRT technique, Barros et al. (20) observed, with this tool support, a correlation between the RT and the surface temperature of buffaloes in hot and humid climates. This technique has also supported the determination of semen quality through the correlation of scrotal surface temperature gradient with sperm quality parameters of buffalo bulls (22, 23).

CONCLUSION

The heat dissipation mechanisms in buffaloes are efficient since, even when submitted to climate conditions that set off thermal stress, the animals were able to regain homeothermy as the environmental variables became milder.

The use of IRT is a good indicator of the BST of buffaloes and its association with thermoregulation. The IRTs of the eye and cheek were the most appropriate to determine thermal stress since they are best correlated with the animals' RT.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee on Experimental Animal Research of the Federal University of Pará under the protocol BIO 120-13.

AUTHOR CONTRIBUTIONS

Experiment design: CB and JS. Experiment perform: CB, JS, LM, and RV. Data curation: CB, JS, and AS. Formal analysis: AS and AVCB. Writing-original draft: CB, JS, ASB, and JL. All authors edited and approved the final manuscript.

FUNDING

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)—Brasil (Finance Code 001) and Brazilian Agricultural Research Corporation—Embrapa Eastern Amazon. Also, this study received financial support for publication fee from Pró-Reitoria de Pesquisa e Pós-Graduação (PROPESP/UFPA). Finally, we would like to thank the Programa Pró-Amazônia: Biodiversidade e Sustentabilidade (Pró-Amazônia/CAPES).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 17 June 2020
doi: 10.3389/fvets.2020.00247

Passive Immunity Transfer in Water Buffaloes (*Bubalus bubalis*)

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This study aimed to evaluate passive immunity transfer in healthy buffalo calves. Colostrum samples from heifers (without previous calving) and primiparous and pluriparous dams and blood samples from their offspring were obtained at calving, before colostrum intake, and at 24, 48, and 72 h after calving for determination of serum activities of gammaglutamyltransferase and alkaline phosphatase and serum concentrations of total protein (TP), immunoglobulin A (IgA) and IgG, and lactoferrin. The results were analyzed as repeated measures, and differences were considered statistically significant at $P \leq 0.05$. Considering that the buffalo calves were born hypogammaglobulinemic (4.23 ± 0.33 mg/ml) and, at 24 h, the mean serum concentration of IgG was 34.5 ± 1.48 mg/ml, passive immunity transfer was successful. Moreover, colostrum IgG concentrations at 0 h were correlated with serum IgG concentrations at 24 h in buffalo calves. Additionally, TP concentrations were highly correlated with IgG in both colostrum at calving and blood in calves at 24 h. TP is recommended as a reliable indirect parameter to evaluate both colostrum quality and passive immunity transfer in buffalo calves.

Keywords: calf, colostrum, murrah, IgG, neonate, newborn

OPEN ACCESS

Edited by:
Domenico Vecchio, Istituto Zooprofilattico Sperimentale del Mezzogiorno, Italy

Reviewed by:
Alessandra Pelagalli, University of Naples Federico II, Italy
Enrico Fiore, University of Padua, Italy

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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *14 January 2020*
Accepted: *15 April 2020*
Published: *17 June 2020*

Citation:
Souza DC, da Silva DG, Fonseca LCC, de Castro Fiori L, Monteiro BM, Bernardes O, Viana RB and Fagliari JJ (2020) Passive Immunity Transfer in Water Buffaloes (Bubalus bubalis). Front. Vet. Sci. 7:247. doi: 10.3389/fvets.2020.00247

INTRODUCTION

The water buffalo (*Bubalus bubalis*) is a species that is important to Asian, Mediterranean, and South American societies because it is essential to the livelihood of many families in developing countries as a source of income and food safety. The most recent reports estimate a world population of 199 million animals, with 193,795,922 heads in Asian countries (1). In South America, Brazil is the largest producer, with 1,381,345 heads, a milk production of 100 million L/year, and an estimated market value of USD 300 million (2).

As ruminants, buffaloes are dependent on immunoglobulin (Ig) present in the colostrum because they are born agammaglobulinemic or hypogammaglobulinemic (3). IgG is responsible for calf immunity during the first month of life and represents 86% of total Igs in buffalo colostrum, with the additional aid of IgA (8%) and IgM (6%) (4). The predominance of IgG is due to active and selective receptors in the epithelium of the mammary gland. These same receptors are present in the intestinal epithelial cells of calves and carry IgG through endocytosis in blood circulation (5).

The most efficient way to ensure low mortality levels in bovine calves is to verify that passive immunity transfer (PIT) occurs, more specifically, that calves have absorbed at least 20–25 mg/ml of colostral IgG. A failure of PIT (FPIT) prevalence of <10% is a reasonable goal in ruminants (6). The odds of FPIT are higher when there is no on-farm routine screening, and benchmarking

PIT values has been shown to enhance production outcomes as better management practices were adopted on dairy farms (7, 8). The measurement of IgG concentrations, both in the colostrum and blood serum of calves, serves as a tool to evaluate colostrum management.

Despite the importance of the species in world dairy production, the available knowledge regarding PIT in buffaloes remains scarce. Therefore, the aim of this study was to evaluate PIT that occurs in newborn buffaloes by measuring serum concentrations of IgG in both colostrum and calf blood and to evaluate associations among the studied parameters.

MATERIALS AND METHODS

Farm Conditions and Animals

Seventy-two healthy Murrah females (15 heifers without previous calving; 18 primiparous buffaloes; 11 multiparous buffaloes with two or three parities; 15 multiparous buffaloes with four or five parities; and 13 multiparous buffaloes with more than six parities), with an average weight after delivery of 627 ± 91 kg, raised in a farm of 116 ha with a semi-extensive system, with an average milk production of 2,650 ± 635 kg in 300 days/dam with an average of 6.40% of milk fat and 4.24% of milk protein, located in São Paulo state, Brazil (23° 34S, 47°49 W) were used in the study. All animals were clinically healthy and had free access to water and *Brachiaria* spp. pastures and were fed with a diet consisting of corn silage, soybeans, and cottonseed twice per day. They were kept under an average environmental temperature of 23 ± 7°C. All females had a dry period of 70 days and were vaccinated against brucellosis, clostridiosis, hemorrhagic septicemia, bovine viral diarrhea, leptospirosis, and foot and mouth disease. No vaccination was done during late gestation.

All their calves (33 females, 39 males; average weight of 38.0 ± 5.55 kg), born from natural breeding via eutocic, were also part of this study. All animals were born in a maternity pen under careful observation to prevent the calves from sucking off another mother. Immediately after deliverance and after the dams recognized their calves, they were separated, and the birth and weight of the calves were registered. After first feeding, the calves' umbilical cords were treated with 2% iodine solution. The calves were free to suckle on their dam during their first 5 days of life. Only calves that had suckled < 4 h after calving entered the study. Animals underwent thorough daily physical examination and were considered clinically healthy when they did not present any alterations on physical examination (9). Sampling of colostrum and calf's blood occurred at calving, before they could suckle (0 h), and at 24, 48, and 72 h after calving. All animals were raised in the same conditions.

Sampling and Chemistry Analysis

A 10-ml blood sample from calves was collected by jugular venipuncture after local antisepsis. A vacuum collection system in siliconized tubes without anticoagulant (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) was used. Blood samples were clotted at room temperature for 30 min, and serum was collected after centrifugation at 2,000 rpm (Excelsa Baby −208N, Fanem, São Paulo, Brazil) on field for 10 min, divided into 2.0-ml aliquots, stored in microtubes, and maintained at −20 °C until the laboratory tests were performed.

The colostrum samples were pooled from all mammary quarters and stored in 50-ml polypropylene tubes put and maintained at −20°C. For colostrum whey separation, renin solution was added (Coalho Estrella, Chr. Hansen Brazil Ind. and Com. LTDA, Valinhos, Brazil) in an amount corresponding to 5% of the volume of milk secretion. The samples were placed in a 37°C water bath for 20 min until formation and retraction of the clot. Subsequently, samples were centrifuged at 5,000 rpm for 20 min in a refrigerated (4°C) centrifuge (Centrifuge 5804 R, Eppendorf, Hamburg, Germany) at the laboratory. After centrifugation, the colostrum whey was aspirated and stored in microtubes, then frozen at −20°C until analysis.

Serum activities of gammaglutamyltransferase (GGT; modified Szasz method) and alkaline phosphatase (ALP; modified Bowers and McComb method) and serum concentrations of total protein (TP; biuret method) were analyzed using a semiautomatic spectrophotometer (Labquest, Labtest Diagnóstica, Lagoa Santa, Minas Gerais, Brazil) with light of appropriate wavelength for each test using a set of commercial reagents (Labtest Diagnóstica, Lagoa Santa, Minas Gerais, Brazil).

Protein fractionation of colostrum and serum samples for the determination of immunoglobulins and lactoferrin was determined using sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), according to the technique described by Laemmli (10). The concentrations of these proteins were determined using computerized densitometry (Shimadzu CS-9301 PC, Tokyo, Japan). As a reference, a marker solution with different molecular weights was used in addition to the purified bovine IgG protein (Sigma, St Louis, MO, USA).

Statistical Analysis

To observe the effects of treatment (dam colostrum or calf blood) throughout the experimental period (0, 24, 48, and 72 h after calving) and the respective interactions (treatment × time), the parameters were analyzed as repeated measures using the MIXED procedure in SAS version 9.4 (SAS/STAT, SAS Institute Inc., Cary, NC, USA). In cases wherein the premise of specificity was not met ($P < 0.05$), the probabilities of time (P-value of time) and interactions of treatment and time (P-value for treatment × time) were corrected using the Greenhouse–Geisser epsilon formula. Comparison between treatments at each time point (P-value) was performed using the least-squares means (LS means) test following repeated measures analysis.

Correlation analysis among all response variables was performed using the CORR RANK procedure in SAS. Relationships among the studied parameters were determined from these correlations. To predict 24-h-old buffalo calf IgG or colostrum IgG (dependent variables), non-polynomial simple regression equations were formulated using other responses as independent variables (colostrum IgG, colostrum TP, and 24-h-old calf TP). For this, the GLM procedure of the SAS program was used using the LS means methodology.

The regression and correlation coefficients obtained were considered high only when determination coefficients (R^2)

≥ 0.70 and correlation coefficients (r) ≥ 0.50. P was considered statistically significant at ≤0.05. Graphics were created using Sigmaplot version 12.0 (Systat Software GmbH, Erkrath, Germany).

RESULTS AND DISCUSSION

Serum concentrations of IgG, IgA, lactoferrin, and TP and serum activities of GGT and ALP in the colostrum and blood of calves are shown in **Figure 1**.

PIT is determined according to colostrum quality and calf absorption. The moment of colostrum intake, the method of administration, and the volume of colostrum are linked to calf absorption, whereas nutrition, breed, calving interval, dry-off period, and vaccination are determinants of colostrum quality. Therefore, IgG concentrations in both colostrum and calf sera should be estimated regularly to test compliance with colostrum management (6, 11).

The most common methods to assess the PIT status in domestic animals, measuring direct IgG concentrations, are single radial immunodiffusion (SRID), used as the gold standard, and enzyme-linked immunosorbent assay (ELISA), even if these methods are not feasibly correlated (12). SDS-PAGE, refractometry, and sodium sulfite or zinc sulfate turbidity test are used to estimate serum IgG concentration based on the overall protein concentration (13). Moreover, there are new on-farm methods, such as the split trehalase IgG assay (STIGA), that are still undergoing validation testing (14).

The mean serum concentration of IgG in the colostrum of dams was 71.4 ± 2.81 mg/ml on first milking, which was a high value considering that 50 mg/ml is used as the standard for good quality colostrum in bovine cattle, as no standard for buffaloes is available (11). This result was higher than those reported for Egyptian buffaloes using SRID (33.20 mg/ml) (15) and Murrah buffaloes using indirect ELISA (54, 51.7 ± 5.99, and 57.9 ± 5.71 mg/ml) (4–6). After calving, IgG concentration decreases as colostrum finishes turning into milk at approximately the fifth day after calving (15).

These results can be linked to the vaccination schedule of the farm, which must be responsible for the high IgG concentrations in the colostrum derived from the dam's bloodstream. Additionally, all buffaloes had good nutrition and had their dry-off period respected, which is important for females to regain body condition until the end of pregnancy (16) and to prepare for all the metabolic challenges inherent in the transition period (17, 18), and so that the mammary gland can recover from the previous lactation. This is essential in primiparous dams, which experience significant physiological changes during their first lactation. Moreover, the division of the females between categories enabled the proper feeding of heifers. Without the dominance of older dams, heifers could eat an amount adequate for growth, calf development, and boosting their immune system status, culminating in high serum concentrations of IgG in colostrum.

Calves were born hypogammaglobulinemic (4.23 ± 0.33 mg/mL) and after suckling colostrum; at 24 h, serum IgG concentrations increased to an average of 34.5 ± 1.48 mg/ml. Similar values have been reported at 24 h in Mediterranean buffalo calves (31.0 ± 2.4 mg/ml) (19) and descendants of Murrah heifers at 24 h (35.3 ± 8.58 mg/ml) (3). Moreover, the values are higher than those measured by indirect ELISA at 6–12 and 12–18 h after first feeding [11.7 ± 0.75 mg/ml (20) and 11.2 ± 0.7 mg/ml (21)].

The high serum concentrations of IgG in calf blood in this study can be attributed to the short time between calving and first feeding (<4 h after birth). As shown in **Figure 1**, there is a point of inflection at 24 h that is common for both colostrum and calf blood serum concentrations, with the IgG concentrations in calf blood surpassing the amount of IgG in colostrum after 48 h. Because calves are allowed to nurse the dam freely, the volume of colostrum ingested by the calf is not limited by human workforce or proper colostrum management, resulting in high serum concentrations of IgG that will endure until the first month of age (3).

The correlation between dam colostrum parameters at calving and calf blood parameters at 24 h is summarized in **Table 1**. A correlation index of 0.46357 ($P < 0.0001$) was found within dam colostrum IgG at parturition and serum concentrations of IgG at 24 h in calf sera.

It has been established that PIT evaluation is better performed between 24 and 72 h after first feeding. However, measurements within 1 week or even 10 days after birth can be performed with reliable confidence (11, 22). A cutoff point of 10 mg/ml for IgG is recommended to determine PIT in dairy bovine calves (23); however, a more realistic standard of 20–25 mg/ml has recently been established for dairy calves based on the improvements made in colostral feeding practices in the dairy industry (24). Unfortunately, there is no cutoff point for PIT in buffalo calves.

Nevertheless, a more efficient PIT means a better average daily gain (ADG) and heavier calves at 30 days in buffaloes (19), with the same positive correlation between serum IgG levels and ADG reported in pre-weaned Holstein calves (25). It is important to strive for higher PIT levels because it clearly enhances production efficiency on dairy farms, contrary to a previous report that there is no benefit in surpassing the recommended IgG threshold for avoiding FPIT (23).

The mean IgA concentration in dam colostrum soon after calving was 9.10 ± 3.89 mg/ml. The present study had higher values at 24 h than those reported in Murrah buffaloes using ELISA (3.22 mg/ml) (4). This difference is probably due to the data sample ($n = 72$ vs. $n = 8$ dams) and method of quantification (SDS-PAGE vs. ELISA).

Calves were born with low serum levels of IgA (2.23 ± 0.14 mg/ml). After feeding, at 24 h, newborns exhibited an average IgA level of 5.41 ± 0.15 mg/ml. IgA acts to prevent infections through agglutination of microorganisms, binding to the intestinal wall receptors and, together with IgG, is essential for providing neonates with immunological protection during at least the first 2–4 weeks of life (26, 27).

The mean lactoferrin concentration in dam colostrum was 6.25 ± 0.30 mg/ml on first milking, which is higher than that of

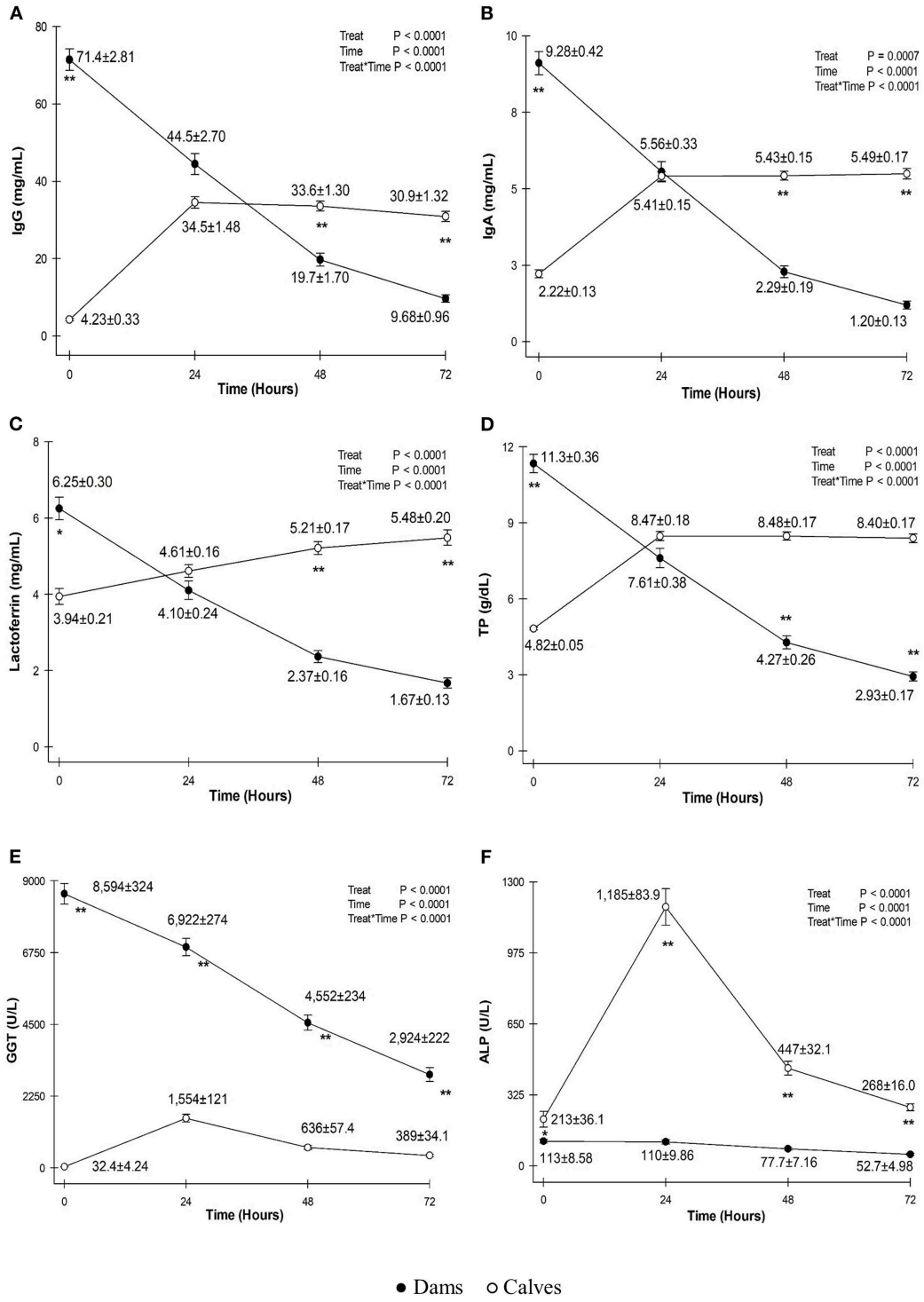

FIGURE 1 | Mean ± standard deviation of serum concentrations of immunoglobulin G (IgG) **(A)**, IgA **(B)**, lactoferrin **(C)**, total protein (TP) **(D)** and serum activities of gammaglutamyltransferase (GGT) **(E)** and alkaline phosphatase (ALP) **(F)** in the colostrum of dams (black circles) and serum of calves (white circles) before colostrum intake and at 0, 24, 48, and 72 h. *P < 0.01; **P < 0.0001.

Egyptian dams (1.08 mg/ml) soon after calving (15). Lactoferrin is present in buffalo milk in lower quantities (0.05–3.40 mg/ml) (28) and is an indicator of the health of mammary glands. It acts as an important immunomodulator because it binds to iron molecules, impeding their availability to bacteria because they bind to cell-surface receptors and facilitate iron absorption (27).

TABLE 1 | Correlation between colostrum parameters at calving and serum concentrations parameters of buffalo calves at 24 h.

	IgGDam	TPDam	GGTDam	ALPDam	IgGCalf	TPCalf	GGTCalf	ALPCalf
IgGDam								
r	1.00000							
P								
TPDam								
r	0.97376	1.00000						
P	< 0.0001							
GGTDam								
r	0.35266	0.37227	1.00000					
P	0.0046	0.0027						
ALPDam								
r	−0.01078	0.04029	0.22487	1.00000				
P	0.9310	0.7462	0.0652					
IgGCalf								
r	0.46357	0.38910	0.14147	0.02769	1.00000			
P	< 0.0001	0.0012	0.2535	0.8174				
TPCalf								
r	0.39218	0.31104	0.07702	−0.02464	0.92647	1.00000		
P	0.0011	0.0110	0.5356	0.8372	< 0.0001			
GGTCalf								
r	0.16585	0.14650	0.49548	0.06960	0.39202	0.34779	1.00000	
P	0.1867	0.2442	< 0.0001	0.5670	0.0008	0.0032		
ALPCalf								
r	0.34998	0.29454	0.21756	−0.02902	0.26533	0.04735	0.17515	1.00000
P	0.0040	0.0164	0.0770	0.8102	0.0253	0.6950	0.1470	

Serum concentrations of lactoferrin in calves exhibited an increase after first feeding, with an average of 4.61 ± 0.16 mg/ml, and continued to increase until the third day (5.48 ± 0.20 mg/ml), as it continued to be absorbed differently from what occurs with Ig's (**Figure 1**). Lactoferrin, when absorbed and in plasma, is called "transferrin" and is responsible for iron transportation. In addition, it has other functions including antiviral and antibacterial activities, and it also acts as a growth factor (27).

We found high correlation indexes between both colostrum IgG and TP at the first milking (0.97376 [$P < 0.0001$]) and between serum concentrations of IgG and TP in calf sera at 24 h after birth (0.92647 [$P < 0.0001$]). Determination indexes are presented in scatter plots in **Figure 2**.

TP is a reliable indirect parameter used to evaluate PIT because TP concentrations have high sensitivity and specificity for the detection of FPIT. Due to the small variation in serum albumin concentrations in newborns, the increase in TP concentrations is almost exclusively due to the absorption of Ig's present in colostrum (29).

Dam colostrum had an average TP concentration of 11.3 ± 2.4 g/dl soon after parturition. TP dynamics, presented in **Figure 1**, show a turning point both in colostrum and calf blood at 24 h, with the TP concentrations in calf sera surpassing those from colostrum and without a significant difference between them ($P \leq 0.05$). Mean serum TP concentration in calves before colostrum feeding was 4.82 ± 0.05 g/dl and, at 24 h of age, reached an average concentration of 8.47 ± 0.18 g/dl, which was above the recommended threshold for an efficient PIT in dairy bovine calves at 24 h (5.8–6.3 g/dl) and consistent with serum TP concentrations reported in buffalo calves born from multiparous buffaloes (3, 24).

In water buffaloes, GGT determines the availability of amino acids for milk protein synthesis during lactation and is present in great quantities during colostrogenesis, accumulating until first milking. In this manner, it has positive correlation (86%) with IgG values measured electrophoretically and is recommended to evaluate colostrum quality from buffalo dams (30, 31). In addition, GGT levels demonstrated the best balance between sensitivity and specificity when compared with IgG measured by ELISA using SRID as the gold standard (29).

Blood serum activities of GGT in calves exhibited an increase between 0 and 24 h (32.4 ± 4.24 to 1,554 ± 121 U/L) following the ingestion of the enzyme present in high quantities in colostrum at the time of calving (8,594 ± 324 U/L). In the following days, serum activities in calf sera decreased, as GGT was degraded over time by the calf intestine (**Figure 1**).

A correlation of 0.49548 ($P < 0.0001$) was found between colostrum serum activities of GGT at first milking and blood serum activities in calves 24 h after birth, which confirms the origin of high serum activities of GGT in calves in the first week of life (3).

The serum activity of ALP in calf blood was low soon after birth and increased at 24 h, with an average of 1,185 ±

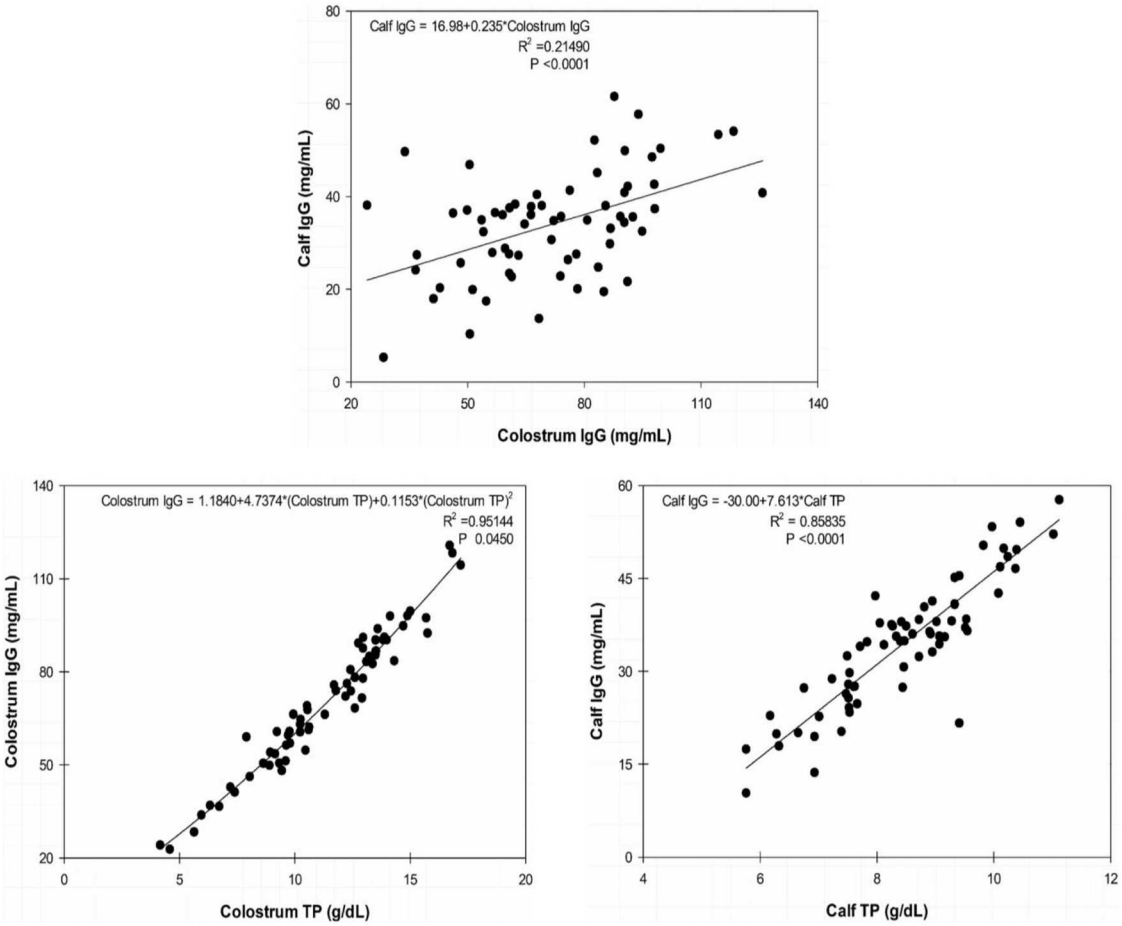

FIGURE 2 | Scatter plots of calf immunoglobulin (Ig)G at 24 h × colostrum IgG at 0 h; colostrum IgG × colostrum total protein (TP) at 0 h; and calf IgG × calf TP at 24 h. The line represents the tendency of the data determined by simple linear regression on calf blood serum and quadratic regression on dam colostrum.

83.9 U/L. This could not be due to absorption of ALP from colostrum because it had low levels since the first sampling (113 ± 8.58 U/L).

There was no correlation between the serum activity of ALP on first milking and calf blood at 24 h. Moreover, a weak correlation was found between the ALP content and the serum concentrations of IgG at 24 h (0.26533 [$P < 0.05$]). The non-correlation of this enzyme with IgG concentrations has been previously reported (30).

The increase in serum activity of ALP might be due to the isoenzyme of bone origin because there is an increase in the activity of bone isoenzyme ALP in animals with high osteoblastic activity, as in neonates (32).

CONCLUSIONS

PIT was successfully achieved in the studied calves. Colostrum from buffalo dams had high IgG concentrations before first milking, which resulted in high IgG concentrations in the calf blood 24 h after birth. Moreover, concentrations of TP served as reliable indirect parameters to evaluate colostrum quality and PIT in buffalo calves. ALP is not recommended for assessing PIT.

Calves had IgG concentrations well above the utilized cutoff point in dairy calves. We emphasize the importance of a vaccination schedule and proper nutrition for dams. Additionally, it is important for calves to nurse directly and freely from the dam. The presence of the dam with the newborn in the first days after calving is also important.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This research project was evaluated by the Ethical Committee on the Use of Animals of FCAV/UNESP, Jaboticabal

Campus (São Paulo, Brazil) and approved under protocol number 17.366/16.

AUTHOR CONTRIBUTIONS

DCS participated in the conception and design of the study, sample collection, laboratory analysis, data interpretation, and coauthored the manuscript. DGS participated in the conception, design and coordination of the study, laboratory analysis, and coauthored the manuscript. LCCF and LCF participated in laboratory analysis and coauthored the manuscript. OB participated in the conception and design of the study, contributed resources, and coauthored the manuscript. BM participated in the conception and design of the study, performed the statistical analysis, and coauthored the manuscript. RV and JF participated in the conception, design, coordination of the study, and coauthored the manuscript. All authors read and approved the final manuscript and study coordination.

FUNDING

This work was supported by Scholarship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior–Brasil (CAPES)–Finance Code 001.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 18 June 2020
doi: 10.3389/fvets.2020.00299

Early Prediction of Corpus Luteum Functionality Using an Imaging Software

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The present study aimed to assess the applicability of luteal blood flow data acquired through the use of color Doppler ultrasonography and a post-processing analysis tool (ImageJ) for predicting pregnancy in buffaloes (*Bubalus bubalis*). The experiment was carried out on 59 multiparous Italian Mediterranean buffaloes that underwent synchronization of estrus and fixed-time artificial insemination (TAI). Corpus luteum features (size: CLS and blood flow: BFA) were taken from Day 5 to 10 after TAI and retrospectively measured with ImageJ. In the same period, blood samples were taken to assess progesterone (P_4) concentrations. Pregnancy diagnosis was carried out on Day 45 by ultrasound and confirmed on Day 70 post-TAI. Differences in CLS, BFA, and P_4 concentrations from Day 5 to 10 after TAI measured between groups were analyzed by ANOVA repeated measures as were differences within each day of measuring. Buffaloes that established a pregnancy ($n = 29$; 55%) had larger CLS (2.2 ± 0.1 vs. 1.9 ± 0.1 cm^2; $P < 0.01$), higher BFA (0.6 ± 0.0 vs. 0.4 ± 0.0 cm^2; $P < 0.01$), and higher P_4 blood level (1.8 ± 0.1 vs. 1.4 ± 0.1; $P < 0.01$) during Day 5–10 as compared to not-pregnant buffaloes ($n = 22$). Throughout the entire period, the first feature that changed between groups was P_4 blood concentration at Day 7 (1.7 ± 0.1 vs. 1.2 ± 0.1; $P < 0.05$) followed by BFA at Day 8 (0.6 ± 0.0 vs. 0.5 ± 0.0; $P < 0.05$), respectively, in pregnant and not-pregnant animals. The ROC analyses indicated that P_4 was able to predict pregnancy since Day 5 ($P < 0.05$) although a more reliable result could be obtained from Day 8 ($P < 0.01$). At Day 10, it was possible to set a cutoff value for every parameter taken into account. The logistic regression analysis showed that pregnancy was positively influenced by P_4 concentration (odds ratio 534.127; $P < 0.01$) and BFA (odds ratio 744.893; $P < 0.01$). In conclusion, the use of color Doppler ultrasonography, together with ImageJ, identified different patterns of BFA between pregnant and not-pregnant buffaloes starting from Day 8 post-TAI.

Keywords: corpus luteum, buffaloes, blood flow area, progesterone, pregnancy diagnosis

OPEN ACCESS

Edited by:
Antonio Minervino, Federal University of Western Pará, Brazil

Reviewed by:
Sarvpreet Singh Ghuman, Guru Angad Dev Veterinary and Animal Sciences University, India
Shankar Yadav, European Commission for the Control of Foot and Mouth Disease (EuFMD), Italy

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *18 March 2020*
Accepted: *04 May 2020*
Published: *18 June 2020*

Citation:
Salzano A, Russo M, Anglani G, Licitra F, Zullo G, Cotticelli A, Fatone G and Campanile G (2020) Early Prediction of Corpus Luteum Functionality Using an Imaging Software. Front. Vet. Sci. 7:299. doi: 10.3389/fvets.2020.00299

INTRODUCTION

The corpus luteum (CL) is essential for establishing and maintaining pregnancy in females (1–3). The advent of color Doppler ultrasound imaging made it possible to examine the intensity of CL blood flow (4) and allows for the generation of real-time information about CL functionality in different phases of the estrous cycle. It is known

that angiogenesis is positively correlated with blood P_4 concentrations during CL development and that the apoptosis of endothelial cells is involved in both functional and structural luteolysis (5). Regarding important features of luteal blood flow, a study demonstrated that the blood flow area (BFA) measured on Day 7 and paired BFA and time average maximum velocity (TAMV) recorded on Day 14 could predict pregnancy in cows (6). Indeed, the most important application for the assessment of CL blood flow is the early detection of not-pregnant females (7–9). A previous study showed, in buffaloes, a strong correlation between CL vascularization and blood flow and P_4 synthesis and release (10, 11). It has also been demonstrated that buffaloes with a greater CL blood flow on Days 10, 20, and 25 after timed artificial insemination (TAI) are more likely to become pregnant, probably due to increased angiogenesis (12). Moreover, Neglia et al. (7) focused on the period between Day 5 and 10 post-AI and revealed that a higher size and vascularization of the CL, together with higher P_4 concentrations in that specific time window, are correlated with pregnancy. In particular, buffaloes with higher CL vascularization on Day 8 after TAI had a higher pregnancy rate, and there is a positive relationship between TAMV values, P_4 concentrations, and pregnancy rates.

The color Doppler ultrasound imaging approach has the potential to improve buffalo reproductive efficiency by allowing for earlier resynchronization of open females and, consequently, a reduction in the interval between services (5, 13). Moreover, the use of a free post-processing tool such as ImageJ could be of great advantage for management strategies that use intensive resynchronization schedules to improve reproductive performance.

Therefore, the present study aims to evaluate the applicability of BFA obtained by using color Doppler ultrasonography and post-processing analyses for predicting pregnancy. Specifically, we addressed questions regarding: (1) changes in CL size (CLS), BFA, and P_4 blood levels from Day 5 to 10 after TAI; (2) how early changes in vascularization, morphology (size), and function of the CL are different in prospective pregnant and not-pregnant buffaloes; and (3) whether vascular image analysis can predict conception using ROC analysis, logistic, and linear regression models.

MATERIALS AND METHODS

The Ethical Animal Care and Use Committee of the Federico II University of Naples approved the experimental design and animal treatments. Written informed consent was obtained from the owners for the participation of their animals in this study.

Animals

The experiment was carried out on 59 multiparous Italian Mediterranean buffalo cows at 133 ± 12 days in milk (DIM) during the breeding season (October–November 2018). Animals involved in the trial were selected from a larger group of buffaloes by a clinical examination that included (1) rectal palpation of the ovaries for follicular development, (2) a functional CL, and (3) no abnormalities of the reproductive tract. The animals were maintained in open yards that allowed 15 m^2 per buffalo and a manger space of 80 cm. The buffaloes were fed a total mixed ration consisting of 55% forage and 45% concentrate, containing 0.91 milk forage units/kg of dry matter and 15% crude protein. Moreover, the body condition score (BCS) of each buffalo was recorded weekly using a 1–9 scale (14).

Estrus Synchronization and AI

Ovsynch protocol with fixed-time AI (TAI) was used to synchronize the animals. This protocol was initially developed in cattle (15) and previously used in buffaloes (16). Briefly, a GnRH agonist (buserelin acetate, 12 mg; Receptal, Intervet) was administered on Day 0, a PGF2α analog (Luprostiol, 15 mg; Prosolvin, Intervet) was administered on Day 7, and a GnRH agonist (12 mg) was administered again on Day 9. Artificial inseminations were performed by the same technician 20 h after the second injection of GnRH. Because of the relatively low estrous behavior in buffaloes (17), animals underwent rectal palpation (immediately before TAI), and only buffaloes considered to be in estrus were inseminated.

Corpus Luteum Development and Blood Flow

Corpus luteum ultrasonography examinations were performed daily from Day 5 to 10 post-TAI by using a portable MyLab Goldvet 30 ultrasound unit (Esaote, Italy) equipped with a 7.5 MHz linear transducer adapted for transrectal examination in large animals. Corpus luteum features (CLS and BFA) were examined daily. To obtain a better definition of the CL, once the ovary was visualized, the image was adjusted and then frozen to measure both the long and short axes, and the color Doppler mode was activated. Color gain setting, velocity setting, and a color-flow filter setting were standardized during all the procedures, and all the analyses were carried out by the same technician. Real-time B-mode/color Doppler images of the continuous scans of the CL were recorded and then analyzed retrospectively. B-mode and color flow mode short video clips (7 s duration) were stored in the internal memory of the ultrasound machine. Later, as described by Siqueira et al. (9), the CL area and the area of the cavity (if present) were calculated using the machine's internal calipers. To justify fluid-filled cavities, CLS was assessed by subtracting the area of the cavity from the entire CL area. To determine the blood flow area, a color Doppler image at the maximum diameter of the CL was examined using an open-source post-processing image tool, such as ImageJ (version 1.49; National Institutes of Health, Bethesda, MD) (18). To assess the area of colored pixels within the CL, an indirect estimation of CL blood flow area (BFA) was made. In particular, the BFA of the corpus luteum was calculated by analyzing the eco-color Doppler images, selecting the colored areas (not including artifacts), and setting the scale (pixels numbers = 1 cm) in each image using the references on the side of the eco-color Doppler images (**Figure 1**).

Progesterone

Starting from Day 5 to 10 after TAI, blood levels of P_4 were assessed by RIA (19, 20) to determine CL functionality. Blood samples from the jugular vein were collected into heparinized

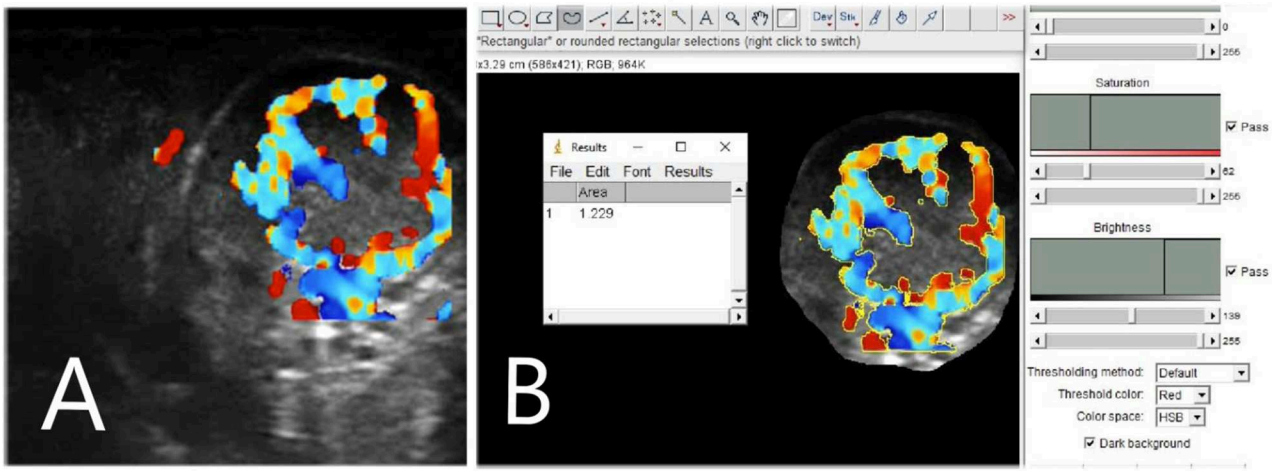

FIGURE 1 | Corpus luteum (CL) blood flow assessment with ImageJ post-processing software. In **(A)** a CL blood flow picture, made with color Doppler mode and in **(B)** the same picture during the post-processing analysis with the software.

tubes on the same days as the ultrasound examinations. The samples were then centrifuged at 800 × g for 15 min and the serum was stored at −20°C until analysis. The minimum detectable amount of P_4 was 2.0 ± 0.09 pg/ml, and the intra-assay and inter-assay coefficients of variation were 6.1 and 11.9%, respectively. Values of 1.2 ng/ml were considered indicative of the presence of a functional CL, according to previous studies (7).

Embryonic Development and Pregnancy

Pregnancy diagnosis was carried out on Day 45 post-TAI by using the same ultrasound machine described above. Pregnancy status was confirmed on Day 70 post-TAI. Animals that were pregnant on Day 45 and not pregnant on Day 70 were considered to have undergone fetal mortality.

Statistical Analysis

All data were presented as the mean ± standard error, and all statistical analyses were performed using SPSS IBM version 22.0 statistical software (21). Differences in DIM and BCS between pregnant and not pregnant were analyzed by the Student *t*-test. Differences in BFA, CLS, and P_4 concentrations from Day 5 to 10 after TAI measured between groups were analyzed by an ANOVA repeated-measures test as were differences within each day of measuring. Statistical significance was determined based on a *P*-value of 0.05. Changes (Δ) in dimensions of CLS, BFA, and P_4 were assessed by subtracting the value of one observation from that of the previous ones: Period 1 (P1, Day 5–6), Period 2 (P2, Day 6–7), Period 3 (P3, Day 7–8), Period 4 (P4, Day 8–9), Period 5 (P5, Day 9–10), and Period 6 (P6, Day 5–10).

Stepwise forward linear regression was performed using the mean of BFA, CLS, P_4, DIM, and BCS, including the differentials (Δ) for CLS, BFA, and P_4, as independent variables to assess their relationships with CLS, BFA, and P_4 in three different groups (all animals A, pregnant P, not pregnant NP). Logistic regression for pregnancy outcome was calculated using CLS, BFA, and P_4 concentrations, DIM, and BCS as independent variables to evaluate whether one of the variables could be used to predict pregnancy. Furthermore, receiver operating characteristics (ROC) analyses were assessed, focusing on three features (CLS, BFA, and P_4 concentrations) on Day 5–10 post-TAI to identify the optimal cutoff value for predicting pregnancy. The latter was determined from the data point minimizing the distance, reporting the relative sensitivity and specificity percentages and area under the curve (AUC) value.

RESULTS

Pregnancy and CL Development and Function

Fifty-nine buffaloes were subjected to synchronization, but only 49 (83%) out of 59 responded to the synchronizing protocol and underwent TAI. On Day 45 and confirmed on Day 70, 27 out of 49 (55%) animals were pregnant. Both DIM and BCS did not differ between groups (134.2 ± 8.4 vs. 127.0 ± 7.8 days and 7.6 ± 0.4 vs. 7.4 ± 0.3, respectively, in pregnant and not-pregnant animals). The average corpus luteum area was similar from Day 5 to 10 in pregnant animals compared to their not-pregnant counterparts (**Table 1**) although a ($P < 0.05$) difference was recorded on Day 5. The first parameter that became different between groups was P_4 at Day 7 ($P < 0.05$), followed by BFA at Day 8 ($P < 0.05$). At Day 10, both CLS and BFA were different ($P < 0.01$) between pregnant and not-pregnant buffaloes (**Table 1**). In terms of differences per each day within the two groups (P and NP), the BFA and the P_4 concentration increased in a time-dependent manner in P group, and in the NP group, the same features started to decrease from Day 9 (**Table 1**).

The average values from Day 5 to 10 post-TAI were greater ($P < 0.01$) in the P group than in the NP group (**Table 2**). In particular, the BFA was 1.5 times higher in the P group than in the NP group, and this was the higher rate compared to CLS and

TABLE 1 | Differences in corpus luteum size (CLS), blood flow area (BFA), and P_4 circulating concentrations between pregnant and non-pregnant buffaloes, during period from day 5 to 10 post-TAI.

Day	CLS (cm^2)		BFA (cm^2)		P4 (pg/ml)	
	P (27)	NP (22)	P (27)	NP (22)	P (27)	NP (22)
5	2.1 ± 0.3^a	1.6 ± 0.1^b	0.3 ± 0.0	0.3 ± 0.0	1.2 ± 0.1	1.1 ± 0.1
6	1.9 ± 0.1	1.8 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	1.5 ± 0.1	1.3 ± 0.1
7	2.2 ± 0.1	1.9 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	1.7 ± 0.1^a	1.3 ± 0.1^b
8	2.3 ± 0.1	2.0 ± 0.1	0.6 ± 0.0^a	0.5 ± 0.0^b	2.0 ± 0.1^A	1.4 ± 0.1^B
9	2.5 ± 0.1	2.2 ± 0.1	0.7 ± 0.0^A	0.5 ± 0.0^B	2.2 ± 0.1^A	1.5 ± 0.1^B
10	2.4 ± 0.1	2.0 ± 0.1	0.8 ± 0.0^A	0.4 ± 0.0^B	2.3 ± 0.1^A	1.4 ± 0.1^B

Values are expressed as mean ± standard error.
[a,b]Values with different superscripts within adjacent rows are different; $P < 0.05$.
[A,B]Values with different superscripts within adjacent rows are different; $P < 0.01$.

TABLE 2 | Differences in corpus luteum size (CLS), blood flow area (BLF), and P4 circulating concentrations mean values from day 5 to 10 post-TAI between pregnant (P; $n = 27$) and not pregnant (NP; $n = 22$) buffaloes.

Group	n	CLS (cm^2)	BFA (cm^2)	P4 (pg/ml)
P	27	2.2 ± 0.1^A	0.6 ± 0.0^A	1.8 ± 0.1^A
NP	22	1.9 ± 0.1^B	0.4 ± 0.0^B	1.4 ± 0.0^B

Values are expressed as mean ± standard error.
[A,B]Values with different superscripts within adjacent rows are different; $P < 0.01$.

P_4 concentration, which were, respectively, 1.16 and 1.29 times higher in the P group than in the NP group (**Table 2**).

Prediction of Pregnancy

The ROC analyses indicated that P_4 was the only predictor of pregnancy, among the others, that was able to detect pregnancy since Day 5 (**Table 3**). Based on the distance from the ideal point, our analysis defined the best P_4 cutoff value as 0.91 pg/ml for the pregnancy prediction on Day 5 (sensitivity 77.8% and specificity 40.9%; $P < 0.05$). On Day 10 after TAI, the P_4 cutoff value was set at 1.82 pg/ml with an increase in sensitivity (85.2%) and specificity (81.8%; $P < 0.01$). Regarding the other parameters, it was possible to set a cutoff value (0.59 cm^2) for BFA only from Day 9 with a sensitivity of 70.4% and a specificity of 59.1% ($P < 0.01$). At Day 10, it was possible to set a cutoff value for every parameter taken into account (**Table 3**).

Regression Analyses

Linear regression analyses of the parameters measured from Day 5 to 10 showed a relationship ($R^2 = 0.571$; $P < 0.01$) only in not-pregnant animals between BFA, Δ5 P4 (D10–D9) and Δ3 P4 (D8–D7) as shown in the equation

$$BFA(cm^2) = 0.456 + 0.196\Delta 5P4(D10 - D9) - 0.131 \Delta 3P4(D8 - D7).$$

The logistic regression analysis showed that pregnancy outcome prediction, from Day 5 to 10 after TAI, was positively influenced by P_4 concentration (odds ratio 534.127; $P < 0.01$) and BFA (odds ratio 744.893; $P < 0.01$). None of the other independent variables (CLS, DIM, BCS) analyzed showed relationships with pregnancy.

TABLE 3 | Summary of the ROC analyses of the three (progesterone, P_4; Corpus luteum area, CLS; Corpus luteum blood flow, BFA) independent variables from day 5 to 10 post-TAI.

Day	Items	Cutoff value	AUC value	Sensitivity (%)	Specificity (%)	*P*-value
5	P_4	0.94 pg/ml	0.71 ± 0.08	81.5	45.5	$P < 0.05$
7	P_4	1.22 pg/ml	0.70 ± 0.07	77.8	40.9	$P < 0.05$
8	P_4	1.43 pg/ml	0.83 ± 0.06	88.9	54.5	$P < 0.01$
9	P_4	1.82 pg/ml	0.80 ± 0.07	81.5	68.2	$P < 0.01$
9	BFA	0.59 cm^2	0.74 ± 0.07	70.4	59.1	$P < 0.01$
10	P_4	1.82 pg/ml	0.91 ± 0.04	85.2	81.8	$P < 0.01$
10	BFA	0.63 cm^2	0.94 ± 0.03	88.9	86.4	$P < 0.01$
10	CL	2.22 cm^2	0.70 ± 0.08	70.4	63.6	$P < 0.05$

DISCUSSION

In this study, we demonstrated that a post-processing tool, such as ImageJ, used to analyze Doppler images could be a suitable tool for studying CL blood flow, whereas the common examinations of the CL area did not seem to be as useful for that purpose. The use of post-processing tools to analyze images was proposed as an alternative to optimize the interpretation of sonographic images of the CL (22, 23) and follicular wall (24, 25). In our study, pregnant buffaloes showed, at Day 10 post-AI, higher BFA and P_4 blood concentration ($P < 0.01$) but not higher CLS compared to their not-pregnant counterparts. This latter finding is in agreement with another study (13), in which no differences in the CL area were recorded during this period (Day 5–10) between pregnant and not-pregnant buffaloes. It is already known that, since Day 5 post-TAI, buffaloes that showed lower CL vascularization had a lower pregnancy rate (7). Indeed, an appropriate BFA is necessary for the release of P_4 into the systemic circulation (26). High progesterone concentration, and particularly an early rise of P_4 during the first days after mating, is necessary to obtain larger embryo development, higher interferon-tau levels, and consequently lower embryo mortality—one of the main causes of reproductive failure in buffaloes (27–29). Moreover, it has already been demonstrated that an appropriate BFA around Day 10 after AI is required to ensure a proper CL function and to increase the likelihood of pregnancy in buffaloes (10).

During this time window (Day 5–10 post-AI), the first feature that changed ($P < 0.05$) between pregnant and not-pregnant animals was the P_4 blood concentration at Day 7, followed by BFA at Day 8. The latter finding confirmed the previous result assessed by several studies performed in buffalo species (7, 30, 31). Because it has already been demonstrated that the use of B-mode ultrasonography alone to monitor CL features had some limits (32), the outcomes of the current study suggest an alternative approach to overcome this problem. Indeed, the evaluation of post-processing BFA by Doppler ultrasonography

could be used, together with the P_4 analysis, as a pregnancy predictor. Moreover, the development of the CLS analyzed by ANOVA repeated measure in both groups did not show any differences throughout the days, and BFA and P_4 showed clear differences at the beginning and end of the trial. Another important result that arose from our study is that, for every analyzed feature, the transition from Day 9 to 10 seems to be a critical period for CL function and could be used to discriminate pregnant and not-pregnant animals. Further studies might be done to investigate the cellular and molecular mechanisms that cause this loss of BFA and CL functionality that started after Day 9 post-TAI in not-pregnant animals. It has already been found (7) that a higher expression of VEGF by luteal cells increases angiogenesis, vasculature development (reflected in increased Factor VIII), and BFA. It could be interesting to study whether the VEGF expression would change from Day 9 to 10 in both pregnant and not-pregnant animals.

To determine the performance of diagnostic tests, we used the ROC curve (33) to set a good cutoff value to predict pregnancy. Even if P_4 blood levels at Day 5 could already give us some important information, more reliable results in terms of sensitivity and specificity could be recorded from Day 8. Moreover, to avoid a stressful condition for the animal, color Doppler ultrasonography and post-processing analyses, such as ImageJ, could also be used. In particular, we found that a BFA of 0.59 cm^2 could be used as a predictor since Day 9 with good sensitivity (70.4%) and specificity (59.1%). However, our findings were in contrast to those of a study performed in cows (6) in which plasma P_4 concentration had limited predictive power for pregnancy compared to BFA. The linear regression for BFA showed that, in not-pregnant animals, it was positively correlated with the P_4 development during the last days ($\Delta 5$ = Day 10–9) and negatively correlated with the same parameter 2 days before ($\Delta 3$ = Day 8–7). This could be explained by the lower P_4 levels produced by the CL during the $\Delta 3$ period. This means that, in not-pregnant animals, the CL is not well-developed, the luteinized phase has not already been completed, and it is difficult for the CL to grow and be vascularized to produce P_4. The logistic regression analysis showed that pregnancy outcome prediction, from Day 5 to 10 after TAI, was positively influenced by P_4 concentration and BFA.

It is worth pointing out that reproductive activity in buffalo species bred in the Mediterranean area is influenced by daylight length (3, 34) and that the breeding season corresponds to late autumn to early winter. Indeed, in sub-tropical zones and at higher latitudes, the reproductive function is mostly influenced by daylight length. During the breeding season, animals showed optimal ovarian function, higher P_4 concentration, and a relatively higher conception rate (2, 30, 34). Our experiment was carried out during the breeding season in order to make our findings suitable at different latitudes (e.g., tropical and equatorial areas) where animals can show estrous cycles throughout the year, influenced mainly by nutrition. In the past, some studies (35, 36) reported that the use of BFA alone was not adequate for the early diagnosis of pregnancy in cattle due to low specificity and sensitivity and to a high variation between animals. However, in recent times, other studies (9, 37) have revealed that these limitations could be overcome because they are due to differences in Doppler ultrasound settings, in the criteria used for the statistical analysis, or in the times when BFA was measured post-AI. In cattle, most of the studies regarding BFA measurement started around Day 20 (32, 35, 37) because only between Day 17 and 21 did BFA decrease in not-pregnant animals due to an enhanced secretion of PGF2α (38). However, Kanazawa et al. (6) indicated that BFA evaluation on Day 7, measured retrospectively with ImageJ, could already be a reliable predictor of pregnancy in recipient cows subjected to embryo transfer. The same findings were reported in a study on bos indicus cattle (39) in which, starting from Day 7, BFA could discriminate pregnant animals from not-pregnant animals. Our findings are in agreement with the latter two studies (6, 39), suggesting that an early BFA measurement could be performed in both buffalo and different bovine species with good results in terms of pregnancy prediction. We are aware that postponing the time window of our analysis could give us better sensitivity and specificity values, but postponing the BFA evaluation also delays the resynchronization of open females. Moreover, there will always be animals that, due to asynchronized ovulations, fail to respond to the TAI or that undergo embryo mortality, giving us false positives.

In conclusion, the use of Doppler ultrasonography, together with a post-processing image tool, such as ImageJ, identified different patterns of BFA between pregnant and not-pregnant buffaloes starting from Day 8 post-TAI. The period between Day 9 and 10 is another important moment because, in those 24 h, there is a switch of all features (CLS, BFA, and P_4) that significantly increase in pregnant animals while they decrease in the not-pregnant counterparts. Considering these findings, both P_4 and BFA measurement seem to be better indexes of luteal function compared to CLS. Thus, BFA measurement at Day 9 or directly at Day 10 could be investigated to detect CL function and predict pregnancy. Moreover, molecular studies that underline the mechanisms of CL development and regression should be deeply studied to understand these phenomena. This will allow for an improvement in the productivity of herds with poor reproductive performance, particularly if early identification of luteolysis is associated with early resynchronization of not-pregnant females.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by The Ethical Animal Care and Use Committee of the Federico II University of Naples approved the experimental design and animal treatments. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

AS and GC: conceptualization. MR and GF: methodology and data curation. GA and GZ: software. FL and GA: validation. AC and GZ: formal analysis. FL: investigation. GC: resources. AS and AC: writing—original draft preparation. GC and MR: writing—review & editing. GF: visualization. GC: supervision and funding acquisition. AS: project administration. All authors contributed to revision of the article for critical intellectual content and have approved the final version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 17 July 2020
doi: 10.3389/fvets.2020.00393

Effect of Silvopastoral Systems in the Thermoregulatory and Feeding Behaviors of Water Buffaloes Under Different Conditions of Heat Stress

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: 23 March 2020
Accepted: 02 June 2020
Published: 17 July 2020

Citation:
Galloso-Hernández MA, Rodríguez-Estévez V, Alvarez-Díaz CA, Soca-Pérez M, Dublin D, Iglesias-Gómez J and Simon Guelmes L (2020) Effect of Silvopastoral Systems in the Thermoregulatory and Feeding Behaviors of Water Buffaloes Under Different Conditions of Heat Stress. Front. Vet. Sci. 7:393. doi: 10.3389/fvets.2020.00393

Buffaloes use wallowing behavior to release excess heat in tropical conditions. The aim of this study was to evaluate the impact of silvopastoral systems in the feeding and thermoregulatory behavior of water buffaloes under moderate and intense heat stress. The behavior of water buffaloes was evaluated in two different production systems. The conventional system with Guinea grass (*Megathyrsus maximus*) only, and the silvopastoral system with Guinea grass and *Leucaena leucocephala* trees. The relation between the frequency of animal activities and the length of time the animals engaged in each activity was measured during the day time (6:00–18:00 h) by visual observations at 10-min intervals. The results obtained suggest that buffaloes use tree shade to partially supplement wallowing. Feeding behavior increased under intense heat stress in the silvopastoral system indicating that it can be a promising alternative to improve the buffaloes rearing conditions in the tropics.

Keywords: buffaloes, silvopastoral system, feeding behavior, thermoregulatory behavior, *Leucaena*

INTRODUCTION

The thermoregulatory behavior of water buffaloes in the tropics includes wallowing in water and shading (1, 2). The reduction in water reservoirs due to climate change can have a negative impact on buffalo production systems where wallowing is essential for thermoregulation (3). Heat stress reduces grazing and as a consequence, a reduction in the productive and reproductive indicators in water buffaloes (4). Previous studies in tropical environments revealed that silvopastoral systems influence the productivity of animals and their response to heat stress (5–7). The combination of leguminous trees and pastures increases 3-fold buffalo production per hectare when compared to systems that do not include trees (8). Trees increase the availability of food (pasture and tree leaves) (9), the nutritional value of grass, and improve soil characteristics by contributing to nitrogen fixation (10, 11). In addition, the inclusion of trees in grasslands has been linked to a decrease in parasitic diseases. For example, the inclusion of *Leucanea* trees reduces infection by *Haemonchus* and *Ostertagia* in buffaloes (12, 13) due to the presence of condensed tannins and polyphenols with antiparasitic effect in these trees (14).

It was suggested, that the use of leguminous trees in silvopastoral systems can replace the need for bathing water, thus becoming a promising alternative in tropical conditions where water is a limiting factor (8, 15, 16). A comparative study showed that a silvopastoral system based on *Leucaena* increased the weight of buffaloes in the early growing stage, a result that was not observed with bovines (17). Furthermore, Iglesias et al. (18) found better average daily weight gain rates for buffaloes in silvopastoralism, without including water for wallowing. This suggests that silvopastoral systems can reduce the need to provide water intended for wallowing to address heat stress.

The thermoregulatory physiology of buffaloes is limited due to the low number of sweat glands per square centimeter and the dark color of their skin that prevents them from thermoregulating efficiently (1, 19, 20). As an evolutionary adaptation, buffaloes express thermoregulatory behavior that includes wallowing in mud or water in flooded zones, or lying down under tree shade (21). Other observations include the movement toward higher quality pasture located near river banks (22) and adjustments of their grazing hours to night hours (23, 24). Research conducted to evaluate alternatives meant to improve thermoregulatory behavior of buffaloes under different conditions, shows that the provision of shade is important (2, 8, 15, 16).

Despite these recent advances, the influence of silvopastoral systems on the feeding and thermoregulatory behavior of buffaloes in tropical conditions under moderate and intense heat stress remains unknown. We hypothesized that the use of trees in silvopastoral systems can reduce wallowing as a thermoregulatory behavior and positively influence feeding behavior. To answer this hypothesis, in this study we examined the influence of the presence of trees on the thermoregulatory behavior of buffaloes under conditions of intense and moderate heat stress. The results show that the use of trees decreases the expression of wallowing as a thermoregulatory behavior and increases the time spent on food consumption.

MATERIALS AND METHODS

Ethics Statement

The experiment recieved the approval of the Scientific Council and the Ethics Committee of the "Indio Hatuey" Grass and Forage Experimental Station, Matanzas, Cuba. This study did not involve any harm or cruelty to the animals.

Study Site and Animals

The study was conducted from 2007 to 2009 in the municipality of Périco, Matanzas, Cuba located at 22° 48'7" of latitude north and 81° 1'of longitude west and 19.01 meters above sea level. The experimental phase was carried out on hydrated red ferralitic soil (25). This soil is moderately acidic [5.60 pH (KCI)], low in phosphorus content (2.43 mg/100 g), contains 0.18% total nitrogen and 3.20% organic matter. Among the exchangeable cations, calcium predominates (11.84 meq/100 g); while the cation exchange capacity (CEC) is slightly low (19.21 meq/100 g), therefore it is considered as having medium fertility. The climate of the region is tropical, seasonally humid, with an annual average temperature between 24.3 and 33.4°C, and a relative humidity of 80%. The annual rainfall was 1,331 mm, where 79.8% of it occurs between May and October (26). Nine female water buffaloes (*Bubalus bubalis*) with an average weight of 167.9 kg and 12 months of age were used for the study. These animals were heifers in 2007, impregnated in 2008 and were lactating in 2009. The animals remained in the pasture during the day and were taken to a paddock at night. In the pasture area, access to water was provided for wallowing. Natural shade by *Dichrostachys cinerea* (Marabú) was available in the wallowing areas. Drinking water and mineral salts were provided *ad-libitum*.

Experimental Design

In this study, the influence of two production systems and different heat stress conditions on the thermoregulatory and feeding behavior of buffaloes were evaluated. The behavior of the animals in two productive systems was compared, the conventional one (i.e., only pastures without the presence of trees, **Figure 1A**) and the silvopastoral (i.e., pastures combined with trees, **Figure 1B**); under two conditions of heat stress, moderate (THI< 75) and intense (THI> 75). The experiment followed the longitudinal analysis method in which thermoregulatory and dietary behaviors were observed in the same group of animals under four different experimental conditions (**Table 1**). Measurements were made in 12-h day cycles for three consecutive days in each experimental condition (T1, T2, T3, and T4). Before each measurement cycle, the botanical composition and pasture availability were analyzed. An adaptation period was included between each 36-h measurement cycle where no measurements were made on the animals for 24 days. At all times, the animals had access to water for wallowing. The total area was 7.33 ha divided into 12 paddocks of an average 0.54 hectare (ha, 10.000 m^2) each. The layout of the experimental area is shown in **Figure 1**.

Description of Production Systems

The conventional system was based on grazing Guinea grass (*Megathyrsus maximus*). The silvopastoral system included Guinea grass and 600 *Leucaena leucocephala* trees per ha. The height of the *L. leucocephala* trees was between 1.7 and 2 m allowing the animals access to the branches as a food supplement. The animals had free access to drinking water and the wallowing area through a central corridor (**Figure 1**).

Determination of Botanical Composition and Pasture Availability

The determination of the botanical composition was done using the step method described by Anon (27) and Sánchez et al. (28), which consisted of walking along the diagonals in each paddock, classifying for every three steps, the species of grass that coincided with the toe of the shoe. The grass with the highest predominance in the system (82–93% of vegetal cover) was *M. maximus*. Other less represented grasses were of the genera *Dichantrium* and *Brachiaria*. The botanical composition and grass availability were determined in order to calculate the grazing pressure [i.e., kg dry mater (DM)/100 kg body weight (BW)] and food offered (i.e., ton DM/ha). The DM of the grass was determined in the laboratory

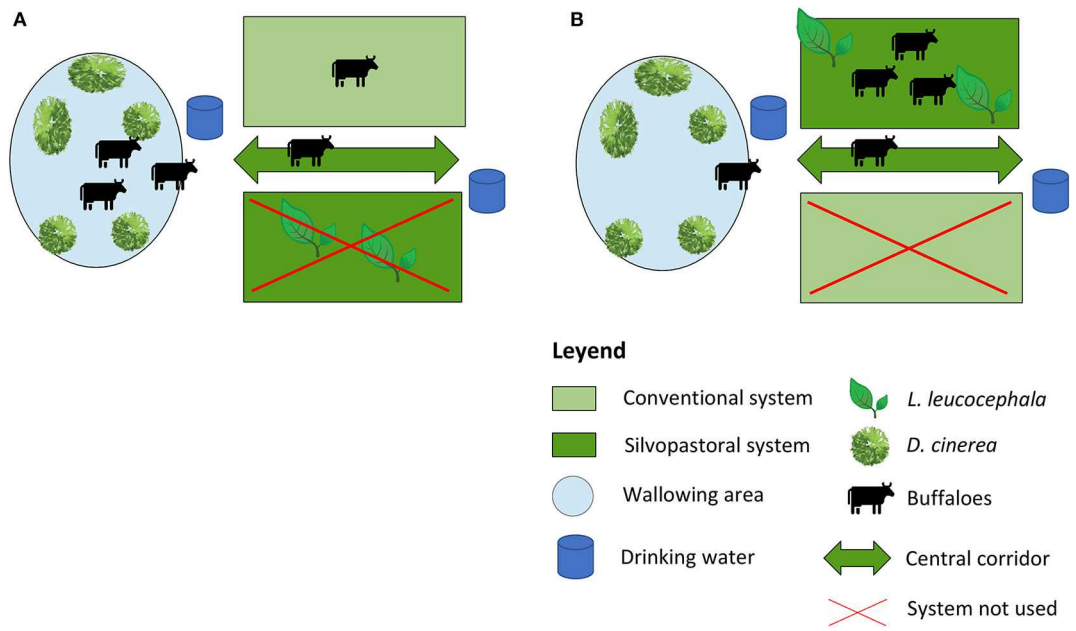

FIGURE 1 | Management of the animals [treeless system **(A)** vs. silvopastoral systems **(B)**] in the study.

TABLE 1 | Outline of experimental conditions.

Treatments	Definition	Number of observations	Frequency (min)	Total time observed (hours)	Number of days	Number of observation cycles
T1	Treeless system in intense thermal stress	1,872	10	312.0	29.0	9.6
T2	Treeless system in moderate heat stress	1,067	10	177.8	16.5	5.5
T3	Silvopastoral system in intense thermal stress	1,361	10	226.8	21.0	7.0
T4	Silvopastoral system in moderate thermal stress	1,226	10	204.3	19.0	6.0

of the Indio Hatuey Grass and Forage Experimental Station. To estimate the DM content, 500 g of the homogenized grass samples was dried in a forced air oven at 60°C for 24 h. The weight was determined before and after the dehydration process (29) using an analytical balance of sensibility 0.001 kg.

The availability of the herbaceous stratum was determined by the visual estimation method using a 0.25 m frame according to the description by Martínez et al. (30). Eighty (80) measurements of grass height were taken in the grazing areas, at random, with a graduated ruler. After the average grass height was obtained, two frames were cut at approximately that height. The following equation was used to calculate availability: DC = (Dm x AA/AM) * 40 where DC: paddock availability, Dm: mean frame availability, AA: mean area height, AM: average height of the frames and factor 40 is the pasture availability within the frame. The initial values of grass availability were homogenized to 2.5 ton DM/ha by slash cutting the area 35 days before the animals entered the paddock.

The density of *L. leucocephala* was determined at the two periods of the year (rainy and dry). The plants encountered in three rows (each row measuring a distance of 30 m) were counted and an average per row was taken. The distance between each row was 10 m. The average number of plants in 30 m was multiplied by the distance between the rows giving rise to the average number of plants per 300 m^2. This was used to determine the density of plants per ha.

Temperature and Humidity Index

The temperature index (ITH) (31) was calculated using the formula (32): THI= (1.8 × T + 32) - [(0.55–0.0055 × RH) × (1.8 × T - 26)] where T is the air temperature (°C) and RH the relative humidity (%). Heat stress was classified as intense (THI> 75) and moderate (THI <75), as reported by Pérez et al. (33) for the conditions of Cuba. The conditions of intense heat stress in Cuba appear between the months of May to October and the conditions of moderate heat stress between the months of November to April. The environmental temperature and relative humidity of the site was measured for each treatment under the conditions of moderate heat stress and intense heat stress at the height of the withers in animals (1.7 m).

TABLE 2 | Description of recorded activities.

Behavior activity	Definition
Active feeding behavior	
Grazing	Time spent eating grass in the paddocks.
Browsing	Time spent in browsing understood as the process of consuming the tips of branches and tree leaves.
Passive feeding behavior	
Rumination	Time spent in rumination understood as the process of regurgitating previously ingested food and masticating it a second time.
Thermoregulatory behavior	
Wallowing	Time spent in wallowing understood as having a bath in the pond of water to cool.
Shading	Performing any activity under the trees (in the systems without trees it was possible in the wallowing area).
Others	
Water intake	Time spent in water intake understood consuming water in the central corridor.

Behavioral Observations

Measurements of feeding and thermoregulatory behaviors of buffaloes were performed using the direct observation method (34). The number of animals in each activity between each measurement interval was recorded. Each observation cycle consisted of 72 observations made over 3 days with a 10-min interval between observations from 6:00 to 18:00 h (**Table 1**). The animals were observed by an experienced observer, who maintained a secure distance which did not influence the behavior of the animals. Several activities were recorded, corresponding to grazing, browsing, rumination, water intake, shading (being positioned in the shade of trees) and wallowing (**Table 2**). The frequency of animals in each behavior per observation was recorded in an Excel database and the time spent on each activity was estimated. The time spent was calculated based on the application of Dumont and Petit (34) equation: Time spent in each activity = sum (ai x n)/A where **ai** is the number of animals that perform the activity, **n** the time between two successive observations and **A** the total number of animals. We then grouped the related variables. Active feeding behavior was considered as the sum of grazing and browsing. Feeding behavior was considered as the sum of active grazing behavior, rumination and water intake. Thermoregulatory behavior was considered as the sum of wallowing behavior and shading behavior (**Table 2**). For more comprensive, we show in **Table 3**, the fodder offered and grazing pressure in Silvopastoral system and conventional system.

Statistical Analysis

The software SPSS® version 25 was used for statistical analysis (IBM Corp®). An analysis of variance (ANOVA) was applied to find the differences between the behaviors, taking into account the levels of intense and moderate heat stress and the type of system (silvopastoral and conventional). The analysis of variance was performed after checking the distribution of normality of the times dedicated to each activity with the Kolmogorov Smirnov test. The average time dedicated to each activity was compared by stress levels and systems with Duncan's multiple range comparison test, in order to detect the inequalities between the means.

TABLE 3 | Fodder offered and grazing pressure in Silvopastoral system and conventional system.

Treatment	Offer of fodder (Dry Matter/ha) per rotation*	Grazing pressure**
T1	4.27 Ton/ha	8.52 kg
T2	2.19 Ton/ha	14.8 kg
T3	6.68 Ton/ha	23.07 kg
T4	3.92 Ton/ha	17.16 kg

*Rotation.
**DM/100 kg body weight.

RESULTS

Conditions of Intense Heat Stress Increase Thermoregulatory Behavior and Decrease Feeding Behavior in Conventional Systems

The wallowing time in the conventional system under conditions of intense heat stress was significantly higher compared to the time of wallowing under conditions of moderate heat stress ($P < 0.05$). In the conventional system, the shading time under intense heat stress differed significantly from the conventional system under moderate heat stress conditions ($P < 0.05$). The grazing time under intense heat stress in the conventional system was significantly less compared to the conventional system under moderate heat stress conditions ($P < 0.05$). The rumination time in intense heat stress was significantly reduced compared to the conventional system under moderate heat stress ($P < 0.05$). Under conditions of intense and moderate heat stress in the conventional grazing system, the time of thermoregulatory behavior is longer under conditions of intense heat stress and differed under conditions of moderate stress. However, water intake, feeding behavior, and active feeding behavior did not differ significantly under conditions of intense and moderate heat stress in the conventional system (**Table 4**). The browsing behavior was not remarkable because in the treeless system they only had occasional contact with some branches of *D. cinerea*, so this behavior did not differ in the conventional grazing system.

The Silvopastoral System Does Not Affect the Global Thermoregulatory Behavior Under Intense Heat Stress

In this study, global thermoregulatory behavior was defined as the sum of time spent in shading and in wallowing and it was measured under conditions of moderate heat stress and intense heat stress. Under moderate heat stress conditions, the shading time did not show significant differences between the silvopastoral system and the conventional grazing system. However, under intense heat stress, differences were found in the time dedicated to shading. Specifically, under conditions of intense heat stress, the time spent dedicated to shading in the

silvopastoral system was higher than the conventional grazing system (ANOVA, $P < 0.05$) (**Table 5**).

Regarding wallowing, under moderate heat stress conditions, no significant differences were found between the silvopastoral system and the conventional grazing system (**Table 5**). However, under conditions of intense heat stress, the wallowing time in the conventional grazing system was significantly longer than in the silvopastoral system (ANOVA, $P < 0.05$).

Overall, the time spent on thermoregulatory behavior under moderate heat stress was greater in the silvopastoral system compared to the conventional system (ANOVA, $P < 0.05$). In contrast, the thermoregulatory behavior of animals in these grazing systems did not differ significantly under conditions of intense heat stress (**Table 5**).

TABLE 4 | Thermoregulatory and feeding behavior in system without trees.

	T1		T2	
	Mean (h)	SD*	Mean (h)	SD*
Thermoregulatory behavior	3.94[c]	6.01	1.14[a]	3.36
Wallowing	1.69[c]	3.08	0.81[a]	2.10
Shading	2.77[b]	3.77	1.61[a]	3.22
Active feeding behavior***	*4.80[a]*	3.92	*5.06[ab]*	3.71
Feeding behavior	*7.78[a]*	2.84	7.59	2.13
Grazing	5.28[a]	3.63	6.12[c]	3.18
Browsing	0.08[a]	0.55	0.01[a]	0.26
Rumination	3.05[b]	3.55	3.70[c]	3.67
Water intake	0.33[b]	1.13	0.32[b]	1.08

[a,b,c]Mean with different superscripts in same row differs significantly for P < 0.05.
**Standard deviation.*
****Active Feeding behavior.*

The Silvopastoral System Increases Active Feeding Behavior Under Intense Heat Stress

The silvopastoral system influenced the time spent on grazing under the two conditions of heat stress. Under moderate heat stress conditions, the time the animals spent grazing was less than in the silvopastoral system compared to the conventional system (ANOVA, $P < 0.05$). In contrast, under conditions of intense heat stress, the grazing time was longer in the silvopastoral system than in the conventional system ($P < 0.05$) (**Table 6**).

The inclusion of *Leucaena* trees in the silvopastoral system increased browsing activity under moderate heat stress and intense heat stress compared to the conventional system where time dedicated to this activity was marginal ($P < 0.05$) (**Table 6**).

In this study, active feeding behavior was considered as the sum of the time spent on grazing and browsing. Under intense heat stress, the animals in the silvopastoral system had significant differences in the active feeding behavior when compared with the conventional system ($P < 0.05$). However, under moderate heat stress conditions, no differences were found between the silvopastoral system and the conventional system (**Table 6**).

Under moderate heat stress conditions, water intake in the silvopastoral system differed from the conventional system (P

TABLE 5 | Time spent in thermoregulatory behavior, wallowing and shading.

Treatment	Thermoregulatory behavior			Wallowing			Shading		
	Mean (h)	SD*	EE**	Mean (h)	SD*	EE**	Mean (h)	SD*	EE**
T1	3.94[c]	6.01	0.13	1.69[c]	3.08	0.07	2.77[b]	3.77	0.09
T2	1.14[a]	3.36	0.10	0.81[a]	2.10	0.09	1.61[a]	3.22	0.14
T3	3.78[c]	5.66	0.15	1.39[b]	2.92	0.08	3.17[c]	3.83	0.11
T4	1.83[b]	4.02	0.11	0.71[a]	1.96	0.06	1.90[a]	3.34	0.11

[a,b,c]Mean with different superscripts in same column differs significantly for P < 0.05.
**Standard deviation.*
***Standard error.*

TABLE 6 | Time spent in active feeding behaviors.

Treatment	Active feeding behavior*			Grazing			Browsing		
	Mean (h)	SD**	EE***	Mean (h)	SD**	EE***	Mean (h)	SD**	EE***
T1	4.80[a]	3.92	0.09	5.28[a]	3.69	0.09	0.08[a]	0.55	0.01
T2	5.06[ab]	3.71	0.11	6.12[c]	3.18	0.10	0.01[a]	0.26	0.01
T3	5.64[c]	4.05	0.1	5.60[b]	3.63	0.10	0.39[b]	1.04	0.03
T4	5.12[a]	3.94	0.11	5.54[ab]	3.48	0.10	0.47[b]	1.37	0.04

[a,b,c]Mean with different superscripts in same column differs significantly for P < 0.05.
**Active feeding behavior is the sum of grazing and browsing.*
***Standard deviation.*
****Standard error.*

TABLE 7 | Time spent in different feeding behavior.

Treatment	Feeding behavior***			Rumination			Water intake		
	Mean (h)	SD*	EE**	Mean (h)	SD*	EE**	Mean (h)	SD*	EE**
T1	7.78[a]	2.84	0.06	3.05[b]	3.55	0.08	0.33[b]	1.13	0.02
T2	7.59[a]	2.13	0.06	3.70[c]	3.67	0.14	0.32[b]	1.08	0.04
T3	8.22[c]	2.33	0.06	2.58[a]	3.58	0.10	0.38[b]	1.18	0.03
T4	7.84[b]	2.25	0.06	3.31[b]	3.74	0.12	0.20[a]	0.87	0.03

[a,b,c]*Mean with different superscripts in same column differs significantly for P < 0.05.*
**Standard deviation.*
***Standard error.*
****Feeding behavior is the sum of grazing behavior, rumination and water intake.*

< 0.05). However, under conditions of intense heat stress, no differences were found between the silvopastoral system and the conventional system (**Table 7**).

Feeding behavior generally showed significant differences under intense heat stress between the silvopastoral system and the conventional treatment. The values found in the active feeding behavior under moderate heat stress conditions also differed significantly between the silvopastoral system and the conventional system ($P < 0.05$) (**Table 7**).

The Silvopastoral System Reduces Rumination Time Under Intense and Moderate Heat Stress

The time spent on rumination in the silvopastoral system under conditions of intense heat stress was significantly different from the conventional system ($P < 0.05$). Significant differences were found for rumination behavior under moderate heat stress between the conventional system and the silvopastoral system ($P < 0.05$) (**Table 7**).

DISCUSSION

Animal behavior studies with water buffaloes in production conditions are scarce and especially important in the tropics. Buffaloes have limited thermoregulatory capacity due to their low number of sweat glands and dark skin which makes them especially sensitive to heat stress (1, 35). In this study, we compared the thermoregulatory and feeding behavior of water buffaloes subjected to intense and moderate heat stress under silvopastoral and conventional systems. In particular, we were interested in evaluating how silvopastoralism influences the behavior of buffaloes under intense heat stress. Describing behavior in the same group of animals in a longitudinal study has as its main strength, the reduction of the individual effect as well as reducing the antagonistic behaviors that occur in the regrouping of the experimental groups in behavior studies (36, 37). However, one limitation of our work is the low number of animals used ($n = 9$).

The greater time spent on wallowing and shading in the conventional system under conditions of intense heat stress suggests an increase in thermoregulation needs (20). It was previously described in Brazil, through infrared thermography measurements, that extreme weather factors (THI> 80) affect buffaloes in the form of heat stress (35, 38). Other studies in India (1) and Thailand (21) also reflect these thermoregulation needs of buffaloes. It is possible that an increase in the thermoregulation needs reflected in the different wallowing and shading times, was due to the seasonality of the reproductive and productive performance of this species (4, 39, 40), as a consequence of the effect of the climatic seasonality present in Cuba and by extension the tropics (8). The decrease in thermoregulatory behavior was associated with a decrease in feeding behavior, which could be related to the heat stress to which the animals were subjected. Previous studies have shown that in intense heat stress, the times dedicated to grazing behavior and rumination decrease as a result of this discomfort in cattle (41).

In the conventional system, intense heat stress resulted in shorter grazing times despite the greater availability of pastures (6, 7, 42). This is relevant because it suggests that under intense heat stress conditions in buffaloes as well as cattle, feeding needs are just as important as heat defense needs (37). This shorter grazing time could also be related to the negative productive results reported in buffaloes in the tropics.

Remarkably, our study revealed that the silvopastoral system decreased the time dedicated to wallowing behavior under intense heat stress. This suggests that the silvopastoral system decreases heat stress in animals and improves their welfare. Previous studies in Cuba attributed these benefits to the microclimate generated under silvopastoralism by mitigating heat stress (5, 6, 18, 43). This was confirmed in our study when it was observed that the decrease in wallowing was associated with a significant increase in the time spent shading.

These results of high active feeding behavior and less passive feeding behavior in the silvopastoral system in heat stress conditions could be attributed to the presence of natural shade (10, 44). *L. leucocephala* and other tree species offer filterable radiation (natural shade). Additionally, the silvopastoral system contributes to a 10–30% increase of dry matter production in the form of grass (5). The different results reported in the productive yield per ha under silvopastoralism and the improvement of animal welfare could be related to those we found in our study. These are, the increase in feeding and thermoregulatory behavior under silvopastoralism and its influence on animal welfare (8, 15, 16, 18, 31, 41, 45, 46); and the decrease in rumination that we observed in the silvopastoral system attributed to the increase in nitrogen supply of the trees that increases ruminal

microbial activity (11, 47), which was reported to be related to an improvement in ruminal function (48, 49).

It is possible that under situations of intense heat stress in silvopastoral conditions, the sensation of heat stress is not being perceived by the buffaloes, due to the contribution of trees in providing shade and improving active feeding behavior (50, 51). In the same way, shade in the silvopastoral system improved pasture quality and quantity in several studies (5, 10, 52).

In the silvopastoral system, under conditions of moderate thermal stress, the time spent on water consumption was reduced. This could be influenced by the composition of the grass (DM) that generate a level of satiety in the animals. Similar results reflected a 23% reduction in visits to the drinking source in cattle provided with shade (41). The similarities in water ingestion under conditions of intense heat stress suggest that the needs for water are more pressing than those for food, coinciding with Dukes (53) and Alvarez et al. (37).

CONCLUSIONS

The silvopastoral system improved the thermoregulatory and feeding behavior under conditions of intense heat stress. This was reflected in the times spent shading and the increased grazing time in comparison with the conventional system where the wallowing time was greater. In the conventional system, the increase in grazing, rumination and wallowing under conditions of moderate heat stress, is a reflection of a greater difficulty to satisfy the thermal and food needs of water buffaloes. In the future, the effect that it would have on the thermoregulatory and feeding behavior of buffaloes without wallowing zones should be evaluated, under silvopastoral conditions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of Experimental Station: Indio Hatuey, University of Matanzas, Cuba. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

MG-H and LS: conceptualization and methodology. MG-H, MS-P, and JI-G: validation. CA-D, VR-E, and MG-H: formal analysis. MG-H, JI-G, MS-P, and LS: investigation. LS, MG-H, and MS-P: resources. MG-H, VR-E, MS-P, and DD: data curation and writing original draft preparation. DD, VR-E, and MG-H: writing—review and editing. MG-H and VR-E: visualization. VR-E and CA-D: supervision. MG-H and LS: project administration. All authors: read and agreed to the published version of the manuscript.

ACKNOWLEDGMENTS

The authors wish to thank Alejandro Cabezas Cruz for his assistance in the redaction and organization of the paper and its concept. We also thank the Indio Hatuey Grass and Forage Experimental Station for its help in the introduction of the buffaloes into silvopastoral systems and in the design of the investigation.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

REVIEW
published: 11 August 2020
doi: 10.3389/fvets.2020.00455

A Review of Toxoplasmosis and Neosporosis in Water Buffalo (*Bubalus bubalis*)

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: 30 April 2020
Accepted: 22 June 2020
Published: 11 August 2020

Citation:
de Barros LD, Garcia JL, Bresciani KDS, Cardim ST, Storte VS and Headley SA (2020) A Review of Toxoplasmosis and Neosporosis in Water Buffalo (Bubalus bubalis). Front. Vet. Sci. 7:455. doi: 10.3389/fvets.2020.00455

Toxoplasmosis and neosporosis are diseases with worldwide distribution that are associated with reproductive problems in livestock and responsible for economic losses. This review presents an overview of the current knowledge relative to these diseases in water buffalo (*Bubalus bubalis*). In general, buffalo are considered resistant to clinical toxoplasmosis because there are studies only reporting serological evidence of natural infection in these animals. Studies have described age, poor hygienic status of the farm, and presence of cats as risk factors for the development of *Toxoplasma gondii* infection in buffalo. It must be highlighted that buffalo meat, which does not receive adequate freezing treatment, could be a potential source for toxoplasmic human infection as well as the importance of raw buffalo milk in the transmission of toxoplasmosis to human beings. *Neospora caninum* is considered one of the major causes of abortion and responsible for huge economic losses in cattle. Vertical transmission is the main route to infect calves, and is responsible for maintaining the parasite within a herd. In buffalo, vertical transmission is also described; moreover, although there are indications that *N. caninum* may be associated with abortion in dairy buffalo, the reproductive importance of neosporosis is apparently lower in buffalo relative to cattle. Most studies have identified a higher time of exposition to *N. caninum* oocysts relative to age. The household system was also described as a risk factor for infection, possibly due to persistent contact between the home-raised buffalo and canids. The fetal immune competence of buffalo is similar to bovine, and buffalo fetus are highly susceptible to infection during the first trimester of pregnancy, indicating that *N. caninum* may be an abortigenic agent in buffaloes. Alternatively, it is interesting to note there is evidence that the inflammatory response in pregnant buffalo infected with *N. caninum* is mild enough to avoid abortion in most cases. It is proposed that the possible transmission of toxoplasmosis through unprocessed milk and buffalo meat may occur, which is important in terms of public health. Additionally, there is strong evidence to suggest that *N. caninum* may be associated with abortion in buffalo.

Keywords: ***Toxoplasma gondii*, *Neospora caninum*, water buffalo, *Bubalus bubalis*, epidemiology**

INTRODUCTION

The domestic water buffalo (*Bubalus bubalis*) is originated from the domestication of the Indian wild buffalo (*Bubalus arnee*), which occurred ∼5,000 years ago (1). Currently, there is an estimated population of 202 million heads, predominantly in Asia (97%), followed by Africa (1.7%) and South America (1%) (2). The buffalo population has been increasing in the last 10 years, probably because these animals are more resistant to infectious diseases and have better converting rates of poor-quality forage into milk and meat than cattle (1–3).

Toxoplasmosis and neosporosis are diseases caused by protozoan *Toxoplasma gondii* and *Neospora caninum*, respectively. These parasites have worldwide distribution and are associated with reproductive problems in livestock and responsible for economic losses (4–6). Buffalo appear to have fewer clinical problems with *T. gondii*, and only serological reports have published; however, it is a zoonotic disease, and attention should be taken relative to the drinking of buffalo milk. Regarding neosporosis in buffalo, although there are several serological studies, the importance of this disease as an abortive agent remains uncertain (7). Therefore, this review presents an overview of the current knowledge of toxoplasmosis and neosporosis in buffalo.

TOXOPLASMOSIS

Introduction

Toxoplasma gondii is a protozoan parasite that is able to infect different animal species, including humans, and has a worldwide distribution (5). Felids are the definitive hosts, being able to shed unsporulated oocysts through feces, which, after sporulation in the environment, can infect many animals, and humans (5). Mammals and birds are the intermediate hosts due to the development of tissue cysts and can be infected by the ingestion of food and water contaminated with sporulated oocysts or by the ingestion of raw or undercooked meat contaminated with tissue cysts (8). Moreover, vertical transmission is an important form of parasitic infection in humans and domestic animals, such as pigs and sheep (9–11). The infection in immunocompetent humans is usually asymptomatic; however, recent studies have reported clinical cases associated with outbreaks (12, 13). In livestock, especially in goats and sheep, toxoplasmosis is responsible for economic losses, mainly due to reproductive disorders, which include abortion, stillbirth, and birth of weak animals (4). The economic impact of toxoplasmosis includes the costs associated with treatment and the reduction in the expected output production (14). In general, buffaloes are considered resistant to the clinical disease, and thus, there are studies only reporting serological evidence of natural infection in these animals.

Epidemiology

Because buffalo meat and milk are used for human consumption, concern relative to the sanitary condition in buffalo herds has increased over the last 10 years (15). Several serological evaluations published worldwide have estimated that the serological status of the contact between buffaloes and *T. gondii* attained prevalence rates ranging from 0 to 87.79%. **Table 1** shows the serological studies for *T. gondii* in buffalo worldwide, and **Figure 1** summarizes the prevalence rates. Different serological techniques, mainly targeting IgG antibodies, for the diagnosis of toxoplasmosis in buffalo were used; these include the indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), modified agglutination test (MAT), Sabin-Feldman dye test (SFDT), latex agglutination test (LAT), and indirect hemagglutination (IHA). An ELISA assay was used to detect the IgM antibody for the diagnosing of acute infection in buffalo (39); however, the effectiveness of this assay is difficult to evaluate because this antibody is maintained only for few weeks after infection (48). The isolation of the parasite performed by mouse bioassay is the gold standard for the detection of *T. gondii* from tissue (5). A study evaluated the diaphragm from slaughter buffalo to isolate *T. gondii*; however, none of the infected mice were positive even those who were inoculated with tissue positive by PCR (46). A bioassay in cats was used to isolate *T. gondii* from the milk of buffalo, demonstrating that this model can be useful to isolate the parasite from different biological samples (49).

Many studies have evaluated risk factors associated with *T. gondii* infection in buffalo (15, 31, 38, 39, 46, 47, 50). A significant difference was observed in the seroprevalence of buffalo from Mexico according to environmental conditions, where water buffalo raised in regions with annual rainfall between 1,266 and 1,650 mm had higher chances to be positive than those raised in regions with higher mean annual rainfall (50). Higher prevalence of IgM antibodies was verified in the monsoon season in Punjab, Pakistan, where the relative humidity, rainfall, and temperature are higher; these are ideal conditions for sporulation of oocysts in the environment (39). Changes in global climatic conditions have already been associated with a possible increase of *T. gondii* prevalence in humans from some regions of Europe (51).

Seroprevalence was considered to be comparatively more elevated in adult buffalo when compared with calves and juveniles (46). Other studies have also described age as a risk factor for the development of *T. gondii* infection in buffalo, where older animals are more seropositive than their younger counterparts (15, 31, 39), probably due to the higher chance of older animals being exposed to the parasite as age increases. Most studies have not observed a significant association between the gender and seropositivity for toxoplasmosis (31, 39, 46, 47, 50); however, a higher prevalence in male buffalo from Iran was observed, but all animals were under 2 years old, the age that was statically associated with infection (38). Conversely, a significant seroprevalence higher in female buffalo from Pakistan was verified with seropositivity being related to a lower immune response during the pregnancy and lactation periods (39).

When the type of rearing system was evaluated, a semi-intensive and extensive rearing system showed higher seropositive animals compared to the intensive system, probably associated with the greater risk of animals ingesting oocysts present in pastures and becoming infected (21, 39). Differently, studies done with buffalo from Romania and Trinidad did not observe any association between rearing systems and the

TABLE 1 | Seropositivity of *Toxoplasma gondii* in buffalo from different countries.

Location							
Continent	**Country**	**Origin**	**Test**	**No. examined animals**	**% positive**	**Cutoff**	**References**
Africa	Egypt	Slaughterhouse	MAT[a]	75	16	25	(16)
	Egypt	Farm	ELISA[b]/LAT[c]	55	74.5/20	n.a.[i]/n.i.[j]	(17)
	Egypt	Slaughterhouse	MAT	160	22.5	25	(18)
	Zimbabwe	Wild (*Syncerus caffer*)	MAT	18	5.6	25	(19)
America	Argentina	Farm	IFAT[d]	500	25.4	100	(20)
	Brazil	Farm	IFAT	136	12.5	64	(21)
	Brazil	Farm	IFAT/ELISA	4796	34.9/40.32	40	(22)
	Brazil	Farm	IFAT	500	38.5	64	(23)
	Brazil	Farm	IFAT/ELISA	321	50.47/42.99	64	(24)
	Brazil	Farm	LAT	104	3.85	64	(25)
	Brazil	Farm	IFAT	169	27.2	64	(15)
	Brazil	Farm	IFAT	374	1.1	64	(26)
	Brazil	Farm	ELISA/IFAT	447	41.6/36.0	64	(27)
	Brazil	Slaughterhouse	IFAT	220	16.8	100	(28)
	Brazil	Farm	IFAT	411	49.9	64	(29)
	Peru	Farm	IHA[e]/IFAT	70	35.7/17.1	16/64	(30)
	Trinidad	Farm	LAT	333	7.8	64	(31)
Asia	China	Farm	IHA	83	0.0	64	(32)
	China	Farm	IAT[f]	40	0.0	n.i.	(33)
	China	Farm	IHA	427	7.5	64	(34)
	China	Farm	IHA	560	16.78	64	(35)
	India	Farm	ELISA	103	2.91	n.a.	(36)
	Iran	Farm	IFAT	385	8.8	16	(37)
	Iran	Slaughterhouse	MAT	300	14.33	25	(38)
	Pakistan	Farm	ELISA	422	13.74	n.a.	(39)
	Pakistan	Abattoir/Farm	LAT	50	14.0	64	(40)
	Philippines	Farm	ELISA	105	1.9	n.a.	(41)
	Vietnam	Abattoirs	DAT[g]	200	3	50	(42)
Europe	Czech and Slovak	Zoo (*Syncerus caffer, Syncerus caffer nanus, Bubalus arnee*)	IFAT	11	18.18	40	(43)
	Czech Republic	Captivity	IFAT/ELISA	5	20/0.0	50	(44)
	Italy	Farm	ELISA	124	13.7	n.a.	(45)
	Romania	Farm/Slaugtherhouse	MAT/ELISA	197	9.64/6.6	6	(46)
	Turkey	n.i.	SDFT[h]	131	87.79	16	(47)

[a] *Modified agglutination test.*
[b] *Enzyme-linked immunosorbent assay.*
[c] *Latex agglutination test.*
[d] *Indirect fluorescent antibody test.*
[e] *Indirect hemagglutination.*
[f] *Indirect agglutination test.*
[g] *Direct agglutination test.*
[h] *Sabin-Feldman dye test.*
[i] *not applied.*
[j] *not informed.*

occurrence of antibodies anti-*T. gondii* in buffalo (31, 46), maybe because of the climatic conditions in these countries.

Other risk factors associated with seroprevalence of toxoplasmosis in buffalo described in the literature are poor hygienic status of the farm and the presence of cats (39). Similar results were described in cattle, demonstrating the importance of cats in the epidemiology of *T. gondii*, because cats are usually common on farms and can shed millions of oocysts through feces, contaminating the environment (14, 52).

Reproductive disorders are usually associated with infection by *T. gondii*; however, there is only one study in water buffalo that analyzed this association, where two (0.6%) out of 307 female

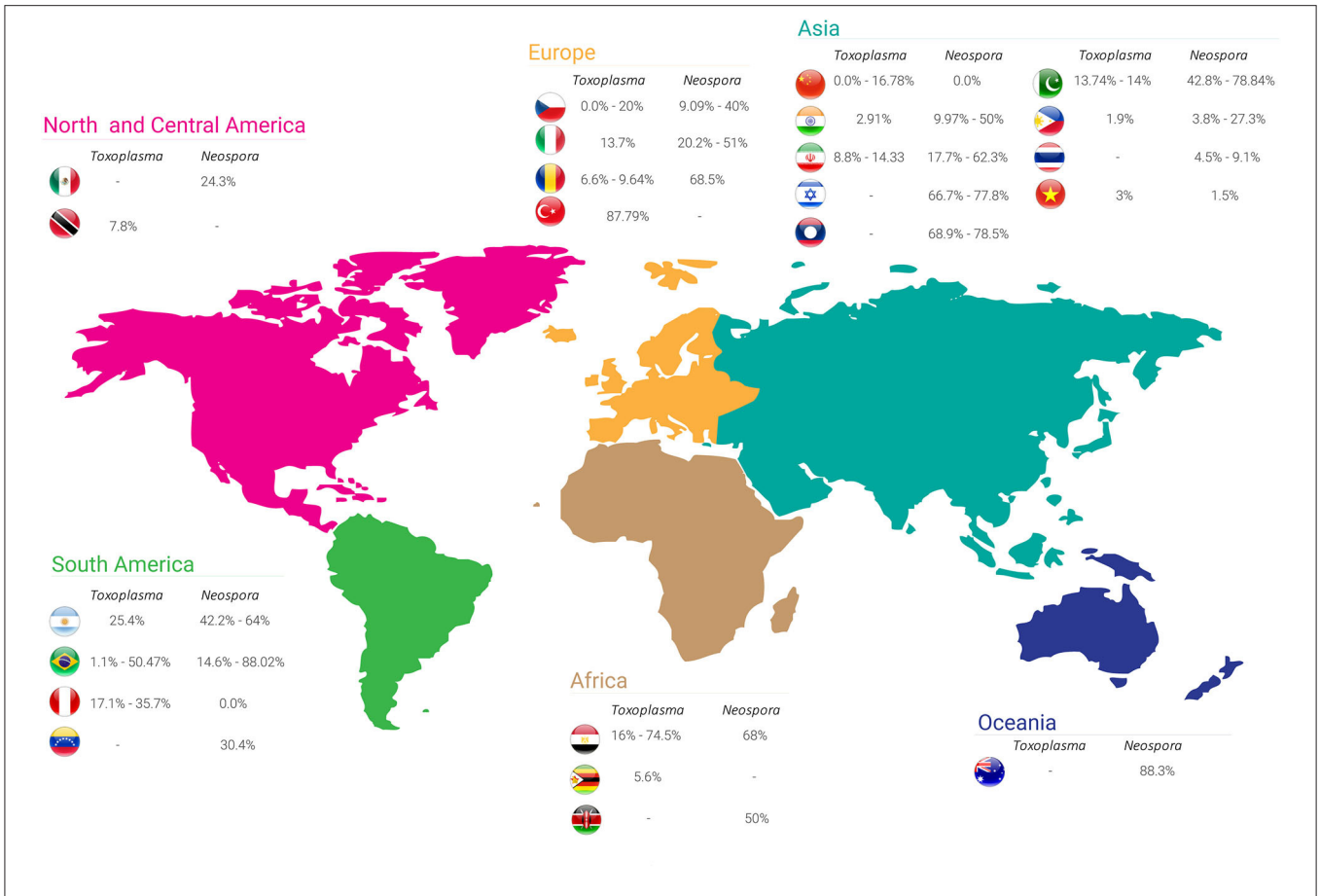

FIGURE 1 | Seroprevalence rates of *Toxoplasma gondii* and *Neospora caninum* in buffalo worldwide.

buffaloes from Mexico had a history of abortion and antibodies against *T. gondii*, resulting in a non-statistically significant association between toxoplasmosis and abortion in water buffalo (50). A recent study reported an association between *T. gondii* infection and a high number of days open (number of days from calving to conception) in water buffalo, and the authors hypothesized that the disease could result in embryonic death and resorption (45).

A study evaluated the presence of antibodies by MAT from 18 wild buffalo, including African buffalo (*Syncerus caffer*) from Zimbabwe, but only one animal was positive with titer of 25 (19). Five African buffalo (*Syncerus caffer caffer*), two Dwarf forest buffalo (*Syncerus caffer nanus*), and four water buffalo (*Bubalus arnee*) from a zoo in the Czech Republic were evaluated by IFAT, but only one African buffalo and one water buffalo were positive with titers of 80 and 640, respectively (43).

Experimental Infections

To the best of the author's knowledge, there is only one study that performed an experimental infection of *T. gondii* in buffalo, and it used eight calves inoculated with a *T. gondii* GT1 strain, five at 10^5 and the other three with 5×10^5 oocysts (53). Five animals developed moderated pyrexia, anorexia, weakness, and dyspnea; one calf inoculated with the higher dose died 11 days post-inoculation (p.i.); and another inoculated with 10^5 had to be euthanized 23 days p.i. because it was unable to eat and stand. Hematological changes were not observed, and the immunological response was first observed on day 21 p.i. by IHA at a cutoff of 64. The antibodies persisted for 63 days and then declined below the cutoff. By mouse bioassay, *T. gondii* was isolated from the brain, lungs, liver, kidney, lymph nodes, and spleen from the calf that died of acute toxoplasmosis. Samples from the lymph nodes and retina were used to isolate the parasite from the calves that were euthanized at 23 and 32 days p.i. These authors reported that the pathological analysis revealed mild interstitial pneumonia in one calf without histopathological evidence of tissue cysts in any of the histological sections (53). The results derived from this study suggest that buffalo, like cattle, are considered resistant to clinical toxoplasmosis and probably do not play an important role in the epidemiology of *T. gondii*.

Public Health Concerns

Although different studies have described seropositivity in buffalo worldwide, the importance of buffalo meat for public health is currently unknown. In cattle, previous studies have not

detected viable parasites in beef from meat stores (54); however, *T. gondii* should be considered an issue of public health in regions where buffalo meat is widely consumed (55). Fresh and imported frozen buffalo meat obtained from markets in Egypt were assessed for viable *T. gondii* by bioassay in cats, and the prevalence of tissue cysts in fresh meat was 15.4% although none of the frozen buffalo meat was considered positive (56). The process of freezing meat at −20°C during importation probably inactivates the tissue cysts because freezing meat to temperature of −12°C or below is efficient in destroying tissue cysts (5).

Toxoplasma gondii tissue cysts were isolated and detected by light microscopy of percoll dilutions in 15% of frozen buffalo meat that was illegally imported to Turkey from Iraq although all positive samples were confirmed by nested PCR using the B1 gene (57). Additionally, the meat was treated at −18°C for 2 days, and tissue cysts were not identified after this process. It must be highlighted that, based on these results, the meat from this study did not receive adequate freezing treatment and could be a potential source for human infection.

Raw milk samples from 33 buffalo herds from Iran were collected from clinically healthy animals and without alterations in physical characteristics of milk for the detection of *T. gondii* (49). Out of 164 milk samples obtained from buffalo, 4.26% were considered positive at Vero cell culture, and 3.65% and 3.04% were positive by capture ELISA and B1 gene PCR, respectively. Additionally, positive samples by the cell culture were used for cat bioassay; animals were fed with 50 ml of milk from buffalo for 3 days, and all challenged cats shed oocysts in the feces, demonstrating viable tachyzoites present in the milk (49). These results demonstrate the importance of raw milk in the transmission of toxoplasmosis to humans.

NEOSPOROSIS

Introduction

Neospora caninum is a protozoan parasite responsible for important diseases in dogs and cattle (58). In cattle, *N. caninum* is considered one of the major causes of abortion and responsible for severe economic losses (6). To date, only domestic dogs (*Canis familiaris*), Australian dingoes (*Canis lupus dingo*), coyotes (*Canis* latrans), and gray wolves (*Canis lupus*) are considered definitive hosts of the parasite (59–62). Although serological positivity against *N. caninum* was reported in several animal species, only a few are considered intermediate hosts, including some mammalian and bird species (58, 63). In humans, the presence of antibodies anti-*N. caninum* was described; however, the zoonotic potential remains unknown (64).

Vertical transmission is the main route of infection in calves and is responsible for maintaining the parasite within a herd (65, 66). In buffalo, vertical transmission was also described, but the importance of this form of transmission in the epidemiology of the disease in buffalo remains to be elucidated. Moreover, the reproductive importance of neosporosis in buffalo has to be clarified; however, this importance is apparently lower than in cattle (7).

Epidemiology

Table 2 shows the seroprevalence of neosporosis in buffalo worldwide with seropositivity ranging from 0 to 88.3% based on different serological assays. A summary of the seroprevalence rates of *N. caninum* in each country is shown in **Figure 1**. Although the seroprevalence in buffalo appears to be three times higher than in cattle, the association between *N. caninum* and reproductive disorders in buffalo seems not to be a common occurrence (6, 7).

Indirect ELISA and IFAT were the main techniques employed for the serological diagnosis of neosporosis in buffalo; however, a competitive-inhibition ELISA (cELISA) and a *Neospora* agglutination test (NAT) were also used. The NAT assay is similar to the MAT assay for the identification of toxoplasmosis; however, it uses *N. caninum* tachyzoites. Moreover, this technique can be done in different animal species because it does not require an anti-IgG conjugate and shows high sensitivity and specificity (99). An indirect ELISA was developed based on fragments of recombinant NcGRA7 for the diagnosis of *N. caninum* infection in buffalo (83). This new ELISA demonstrated 98.6% sensitivity and 86.5% specificity using buffalo sera, obtained a good correlation with a commercial ELISA, and seems to be a good tool for the serological screening of neosporosis in buffalo.

The presence of an immunodominant 17–18 kDa antigen by Western blot was used to confirm the presence of antibodies anti-*N. caninum* in buffalo from Israel (86). Western blot confirmed infection in 85.7% (12/14) of the positive samples by MAT and 84.6% (11/13) by IFAT, demonstrating high sensitivity for both techniques (86). Recently, a milk ELISA was evaluated as an alternative method to detect *N. caninum* antibodies in lactating buffalo (91). The milk iscom ELISA detected a comparatively reduced number of positive milk samples than competitive ELISA using serum samples; however, a moderate agreement between the techniques was observed, indicating that this milk ELISA could be used in lactating dairy herds with a high prevalence of neosporosis (91).

Bioassay in dogs, gerbils, and cell culture were done to isolate *N. caninum* from brain tissues of six buffalo seropositive by IFAT (cutoff = 100) (100). Four dogs shed oocysts through feces after being fed buffalo brain, the higher number of oocysts shed was 820,655, and the higher duration of oocyst shedding was 26 days. These authors report that, by cell culture, *N. caninum* was isolated from the brains of two buffalo, and via bioassay in gerbils, the parasite was isolated from the brains of three buffalo. Additionally, the isolates, named NCBrBuf-1 to NCBrBuf4, were confirmed as *N. caninum* by PCR and DNA sequencing, targeting the Nc5 and ITS-1 region, and showed a low degree of genetic diversity between other *N. caninum* isolates (100). Collectively, these results demonstrate that buffalo are natural intermediate hosts for *N. caninum*; this study is the first report of isolation of the parasite in this species (100).

A recent study revealed a higher seroprevalence for *N. caninum* in adults relative to calves and young buffalo (97). This result is in accordance with previous studies (20, 68, 69, 82, 87, 95) and may be related to a comparative higher period

TABLE 2 | Seropositivity of *Neospora caninum* in buffalo from different countries.

Location							
Continent	**Country**	**Origin**	**Test**	**No. examined animals**	**% Positive**	**Cutoff**	**References**
Africa	Egypt	Slaughterhouse	NAT[a]	75	68	20	(16)
	Kenya	Game ranch (*Syncerus caffer*)	NAT	4	50	40	(67)
America	Argentina	Farm	IFAT[b]	449	64	100	(68)
	Argentina	Farm	IFAT	500	42.2	100	(20)
	Argentina	Farm	IFAT	1,350	43.3	100	(69)
	Brazil	Farm	IFAT	136	19.1	200	(21)
	Brazil	Farm	IFAT/ELISA[c]	47,96	48.8/55.55	100	(22)
	Brazil	Farm	IFAT	500	39	128	(23)
	Brazil	Farm	IFAT	345	35.4	100	(70)
	Brazil	Farm	IFAT/NAT	222	64/53	25/40	(71)
	Brazil	Farm	IFAT	196	70.9	25	(72)
	Brazil	Slaughterhouse	IFAT	220	36.4	100	(28)
	Brazil	Farm	IFAT	374	40.9	200	(26)
	Brazil	Farm	IFAT/ELISA	288	53.12/17.36	200	(73)
	Brazil	Farm	ELISA	164	14.6	n.a.[f]	(74)
	Brazil	Farm	ELISA	192	88.02	n.a.	(75)
	Brazil	Farm	IFAT	411	56.0	200	(29)
	Mexico	Farm	ELISA	144	24.3	n.a.	(76)
	Peru	Farm	IFAT/ELISA	83	0.0	100	(77)
	Venezuela	Farm	ELISA	174	30.4	n.a.	(78)
Asia	China	Farm	ELISA	40	0.0	n.a.	(33)
	India	Farm	cELISA[d]	32	50	n.a.	(79)
	India	Farm	cELISA	341	9.97	n.a.	(80)
	India	Farm	cELISA	51	21.6	n.a.	(81)
	Iran	Slaughterhouse	ELISA	181	37	n.a.	(82)
	Iran	Farm	ELISA	122	62.3	n.a.	(83)
	Iran	Abattoir	ELISA	83	19.27	n.a.	(84)
	Iran	Farm	ELISA	236	17.7	n.a.	(85)
	Israel	n.i.[g]	MAT/IFAT/WB[e]	18	77.8/72.2/66.7	200/200/20	(86)
	Laos	Farm	ELISA	130	78.5	n.a.	(87)
	Laos	Farm	ELISA	61	68.9	n.a.	(88)
	Pakistan	Farm	cELISA	300	54.7	n.a.	(89)
	Pakistan	Farm	cELISA	312	42.8	n.a.	(90)
	Pakistan	Farm	IFAT	52	78.84	200	(91)
	Philippines	Farm	ELISA	105	3.8	n.a.	(41)
	Philippines	Farm	cELISA	176	27.3	n.a.	(92)
	Thailand	Farm	IFAT	628	9.1	100	(93)
	Thailand	Farm	ELISA	532	4.5	n.a.	(94)
	Vietnam	Farm	IFAT/ELISA	200	1.5	640	(42)
Europe	Czech and Slovak	Zoo (*Syncerus caffer, Syncerus caffer nanus, Bubalus arnee*)	IFAT	11	9.09	40	(43)
	Czech Republic	Captivity	IFAT/cELISA	5	40.0/20.0	50	(44)
	Italy	Farm	IFAT	1,377	34.6	200	(95)
	Italy	Farm	ELISA	908	51.0	n.a.	(96)
	Italy	Farm	ELISA	124	20.2	n.a.	(45)
	Romania	Farm/Slaughterhouse	ELISA	197	68.5	n.a.	(97)
Oceania	Australia	Farm	ELISA	480	88.3	n.a.	(98)

[a] *Neospora caninum agglutination test.*
[b] *Indirect fluorescent antibody test.*
[c] *Enzyme-linked immunosorbent assay.*
[d] *Competitive-inhibition enzyme-linked immunosorbent assay (cELISA).*
[e] *Western blot.*
[f] *not applied.*
[g] *not informed.*

of exposition to oocysts relative to age. A higher prevalence in older buffalo (more than 10 years old) was verified, suggesting that horizontal transmission occurs more commonly in buffalo than vertical transmission (93). Another study demonstrated an association between age and *N. caninum* seroprevalence by IFAT with higher prevalence in animals that were 6–11 years old; however, this association was not observed when these samples are analyzed by NAT (71). Alternatively, previous studies from Brazil (72), India (80), and Thailand (94) did not demonstrate statistical differences among age-related groups and neosporosis. When the gender of buffalo was compared, differences in infection by *N. caninum* were not identified between male and female in studies from Argentina (68), Thailand (93), and Pakistan (89, 90); however, serological surveys done in Iran (82) and Romania (97) showed statistical differences for gender with higher prevalence in female buffalo.

In female water buffalo from Brazil, the risk factors associated with neosporosis were grazing and animals derived from animal markets and/or reputable sellers (70), reinforcing the hypothesis of postanal transmission due to the ingestion of food or water contaminated with oocysts. By logistic regression analysis, the presence of pigs was considered as a risk factor for neosporosis in buffalo in Paraíba state, Brazil (21), although pigs do not play an important role in the epidemiology of *N. caninum*. The household system was also described as a risk factor by *N. caninum*, probably due to the persistent contact between home-raised buffalo and canids (97).

Contact with dogs was associated with infection in buffalo from Pakistan, suggesting the role of dogs in contaminating buffalo feed, resulting in horizontal transmission (89). In this same study, summer weather was also associated with seroprevalence of *N. caninum* in buffalo, and it was suggested to be related to the gestational phase of these animals because, during the Pakistan summer, most animals are in midgestation, which could result in changes in the immune system and parasite transmission (89).

Although reproductive disorders, such as abortion, appear to be less common in buffalo relative to cattle, the prevalence of *N. caninum* antibodies was significantly higher in dairy buffalo that had a history of abortion than those without any abortive history, indicating that this agent may be associated with abortion in dairy buffalo (89). It was reported that, in 167 buffalo seropositive for *N. caninum*, 13.2% (22/167) were co-infected with *Brucella abortus*, indicating a higher abortion risk in these animals than if infected with only one of these pathogens (90). Similarly, *N. caninum* infection was associated with abortion and retained fetal membranes, indicating that neosporosis can be related to reproductive disorders in water buffalo (45).

Neospora-like cysts were observed in the brain of the fetuses of two buffalo by histological analysis on a farm in Italy that had a history of abortion and death of buffalo calves with neurological manifestations (95); however, additional confirmation of the identity of the intralesional cysts by other diagnostic methods, such as immunohistochemistry on *in situ* hybridization, was not done to confirm these tissue cysts as *N. caninum* (95). It was demonstrated that, in 17 calves born from seropositive dams, antibodies anti-*N. caninum* persisted for 12 months, suggesting neonatal transmission of the parasite in buffalo, probably by the transplacental route (101). However, the first evidence of congenital transmission of *N. caninum* in buffalo was suggested in a 3-month-old fetus that was negative at IFAT and brain tissue positive by a nested PCR assay that targeted the ITS1 and Nc5 regions (102). These conflicting results could be due to the immunocompetency of the fetus (102).

DNA from *N. caninum* was detected by qPCR in the fetus of one water buffalo from a fetus from a dam that was 2–3 years old, during which abortion occurred between 180 and 210 days of gestation (103); however, the authors concluded that they could not have confirmed if neosporosis was the only cause of abortion because other abortive agents could be present. Consequently, they suggest that *N. caninum* could be responsible for reproductive failure in water buffalo. *N. caninum* DNA was identified by 18S PCR assay in the brains and hearts of three buffalo fetuses that suffered spontaneous abortion between the fourth and 6 months of gestation (96). Additionally, these fetal tissues were considered negative for the major pathogens responsible for reproductive failures in buffalo, including *Brucella* spp., *Salmonella* spp., *Chlamydophila* spp., *Listeria* spp., *Campylobacter* spp., *Coxiella burnetti, Leptospira* spp., *Toxoplasma gondii*, Bovine and Bubaline Herpesvirus (BoHV1 and BuHV1), and bovine viral diarrhea virus (BVDV), indicating *N. caninum* as an important abortive agent (96). In a recently published study, a *Neospora*-associated abortion was reported for the first time in buffalo from India, where tissues from two fetuses were positive by immunohistochemistry (81).

The presence of the parasite DNA was detected by nested PCR assay only at the diaphragm (heart and mesenteric lymph node were also evaluated) from six animals (8.1%, 6/74) of different ages; three of these animals also were negative by serological assays (97). These results demonstrate that the diaphragm may be an adequate tissue for the detection of *N. caninum* in buffalo and that serologically negative animals can also have the parasite in their tissues.

Recently, it was demonstrated that buffalo with higher titers of antibodies against *N. caninum* also contained parasite DNA in the serum samples, being the first report to confirm *N. caninum* DNA in serum samples of buffalo and indicating that parasitemia occurs simultaneously with antibody production (84).

Experimental Infections

The first experimental study with *N. caninum* in buffalo was performed in 2005 when the authors inoculated six buffalo with 5×10^6 tachyzoites of Illinois strain by subcutaneous route (101). All animals were positive between 7 and 11 days p.i. by IFAT with a cutoff of 25, and titers peaked at 3 weeks p.i. and remained elevated until 7 weeks p.i., after which the peak started to decrease (101).

Intravenous inoculation of 10^8 tachyzoites of the NC-1 strain was performed in an experimental study involving 10 female water buffalo at 70 ($n = 3$) or 90 ($n = 7$) days of pregnancy (104). Although all dams developed specific antibodies with titers ranging from 3,200 to 51,200, none of these showed clinical signs or aborted; however, one non-viable fetus was observed from a female inoculated at 70 days of gestation and slaughtered

at 28 days p.i. (104). Specific antibodies against *N. caninum* (cutoff = 10) were detected in only two fetuses; both dams were inoculated at 90 days of gestation and slaughtered at 72 days p.i. Moreover, *N. caninum* DNA was detected by nPCR in the placenta and fetuses of all inoculated animals (104). These results indicate that fetal immune competence of buffalo is similar to that of cattle at ∼110 days of gestation, and fetuses of buffalo are highly susceptible to infection during the first trimester of pregnancy, indicating that *N. caninum* should be considered as an abortigenic agent in water buffalo.

An experimental infection was done to evaluate the outcome of infection with different strains of *N. caninum* in six pregnant buffalo at the 70th day of gestation with 5×10^8 tachyzoites by intravenous route (105). Three pregnant buffalo with the NC-1 strain and the others with the NC-Bahia, a Brazilian strain isolated from the brain of a naturally infected dog. Additionally, all the females developed antibodies detected by ELISA and IFAT (cutoff = 50), but none of the fetuses were considered seropositive (cutoff = 25). Moreover, fetal death occurred in all females infected with the NC-1 strain but not in dams inoculated with the NC-Bahia strain. Furthermore, a semi-nested PCR detected *N. caninum* DNA in maternal and fetal tissues, indicating transplacental transmission in all animals, but the NC-Bahia seems to be less virulent for pregnant buffalo (105).

Pathogenesis

Neospora caninum is recognized as one of the major causes of reproductive failures in cattle; however, little is known about the pathogenesis of the disease in buffalo. So far, natural abortion in buffalo by neosporosis was only reported in one study (96), and all knowledge relative to the pathogenesis is based on experimental observations.

Non-suppurative encephalitis and myocarditis were observed in aborted fetuses, and few *N. caninum*–like cysts were identified in histologic sections of the brain, but none of the molecular techniques were used, compromising the diagnoses as *N. caninum* (95).

Challenged pregnant female buffalo with *N. caninum* tachyzoites developed non-suppurative placentitis as the more frequent histopathologic finding, being observed in 9 out of 10 specimens (104). Additionally, meningoencephalitis, characterized by gliosis and non-suppurative inflammatory infiltration at the pia mater with multiple necrotizing foci at the central nervous system, were observed in fetuses derived these female buffalo. Moreover, in other fetal tissues, including the lungs, heart, kidneys, striated muscle, and liver, multiple foci of mononuclear inflammatory cells were observed in almost all samples analyzed, and immunohistochemistry identified intralesional tachyzoites in two fetuses that were associated with focal necrosis and gliosis in the central nervous system in one of these (104).

Non-suppurative inflammation in placentomes and fetal tissue was also observed after infection of nine pregnant buffalo in early gestation with the NC-1 strain of *N. caninum* (106). Moreover, a mononuclear inflammatory infiltrate was identified surrounding the necrotic foci at the caruncle or within necrotic fetal villi in the placentome of all dams challenged at 70 days of gestation with 10^8 tachyzoites of the NC-1 strain of *N. caninum* and culled at 28 days p.i. with higher scores in dams carrying non-viable fetuses. These authors suggested that the inflammatory response in pregnant buffalo infected with *N. caninum* was mild enough to avoid abortion in most of the cases (106).

Lymphocytic infiltration was the main lesion observed in placental and fetal tissues after pregnant buffalo were inoculated with two strains of *N caninum* (105). Hemorrhagic hepatitis and nephritis were observed in the fetuses, and necrosis at the placenta and fetal brain were also observed. These authors indicate that the fetuses from the dam inoculated with the NC-Bahia strain contained fewer lesions with comparatively less severity when compared with fetuses inoculated with NC-1 strain, indicating that the NC-Bahia strain is more pathogenic than the NC-1 strain of *N. caninum* (105).

Mild satellitosis of the brain and perivascular macrophage at the heart were observed in two pregnant buffalo inoculated with NC-1 tachyzoites (75). Additionally, periportal non-suppurative hepatitis with centrilobular infiltration of macrophages and perivascular mononuclear cell infiltration at the kidney was also observed in these females. Moreover, brain tissues from fetuses from these pregnant buffalo presented multifocal necrosis and gliosis associated with infection by *N. caninum*, and the fetuses revealed non-suppurative myocarditis and pericarditis with macrophage infiltration, myositis, lymphocytic and macrophagic portal hepatitis, and a non-suppurative nephritis with perivascular and periglomerular lymphocytic and macrophage infiltration. Furthermore, the kidney of one of the fetuses had hemorrhage in the renal pelvis, and lung tissue from other fetuses showed peribronchiolar lymphocytic and macrophage infiltration with multifocal hemorrhage (75).

CONCLUSIONS

The buffalo industry is growing worldwide, and the review of the literature indicates that the possible transmission of toxoplasmosis through either unprocessed milk or buffalo meat may occur. Furthermore, considering the high *T. gondii* seroprevalence observed in these animals, we could consider that the public health risk incurred by persons exposed to infected animals is probably higher than previously assumed. We summarized all seroprevalence studies of *N. caninum* in buffalo, which showed a high prevalence worldwide Additionally, there is strong evidence to suggest that *N. caninum* may be associated with abortion in buffalo. Finally, the present review will contribute to lightening future studies in this field.

AUTHOR CONTRIBUTIONS

LB, JG, KB, SC, VS, and SH contributed to conception, design, drafting, and critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

JG, KB, and SH are recipients of CNPq fellowships.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 11 August 2020
doi: 10.3389/fvets.2020.00432

Study of Milkability and Its Relation With Milk Yield and Somatic Cell in Mediterranean Italian Water Buffalo

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Milkability is defined as the ability of an animal to give a regular, complete, and rapid milk secretion by the mammary gland in response to a proper milking technique. The aim of the present study was to investigate the relationship of milkability pattern with milk yield and somatic cell score in buffaloes. Milk yield and milkability can be observed through the milk flow profiles recorded by an electronic milkmeter (Lactocorder). A total of 2,288 milk flow curves of Mediterranean Italian buffaloes were used for one-way analysis of variance, and eight milk emission patterns were studied. The most represented milk flow curve was type 3 (27.32%), followed by type 6 (17.79%) characterized by a very long plateau phase. The less represented curve was type 1 (4.41%) characterized by long lag time and low peak flow rate. According to analysis of variance, milk yield ranged from 2.21 to 5.22 kg per milking for types 1 and 6, respectively, whereas the peak flow rate was minimum (0.50 kg/min) and maximum (1.73 kg/min) for types 1 and 4, respectively. The total milking time was on average 11.29 ± 3.68 min; lag time and milk emission time averaged 2.19 ± 2.34 min and 4.30 ± 2.33 min, respectively. The 12.5% ($n = 286$) of total curves were classified as bimodal and 60 of these were found in type 4. Based on literature, type 4 curves are representative of very short teat canals and very high milk flow. Average somatic cell score was 3.63 ± 1.67 units, with maximum least-squares mean found for type 1 and minimum for type 6. Buffaloes showing curves of type 5 and 6 were characterized by the greatest milk yield at milking, lowest somatic cell score, and lowest milking time. Results of the present study evidenced that such traits could be used in the dairy buffaloes as indicators to improve udder health and milkability.

Keywords: milk flow curves, milk yield, milking time, economic approach, somatic cells

OPEN ACCESS

Edited by:
Selwyn Arlington Headley, State University of Londrina, Brazil

Reviewed by:
Arda Yildirim, Gaziosmanpaşa University, Turkey
Mohanned Naif Alhussien, National Dairy Research Institute (ICAR), India

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *19 April 2020*
Accepted: *16 June 2020*
Published: *11 August 2020*

Citation:
Boselli C, De Marchi M, Costa A and Borghese A (2020) Study of Milkability and Its Relation With Milk Yield and Somatic Cell in Mediterranean Italian Water Buffalo. Front. Vet. Sci. 7:432. doi: 10.3389/fvets.2020.00432

INTRODUCTION

The buffalo population in the world was about 206 million heads in 2018, of which the 97.57% were raised in Asia (1). European buffaloes belong to the water buffalo species *Bubalus bubalis* and are farmed for dairy. Buffalo milk is an important source of energy and nutrients for the human diet in some countries, owing to a particularly high fat content (7–8%) compared with bovine milk (2). In Italy, the Mozzarella di Bufala Campana cheese is one of the most important Protected Designation of Origin foods in terms of internal market demand and export.

Monitoring the milkability of animals allows to improve efficiency of milking procedures and reduce farm production costs (3, 4). More than 50% of the working time in a dairy farm is required for milking and thus has a great impact on the farmers' profit (5, 6). To optimize milking time, farmers should select the most adequate animals or group animals with similar characteristics in terms of milkability. Having buffaloes with good milking properties is crucial for an optimal milking process, such as start milking, milk flow, uninterrupted milk flow curve, and duration of milking.

Milk production and milk flow profiles are important parameters to be recorded and evaluated, as may be informative of milking management (7). Graphical representation in milk ejection is visible through flow curves, which differ according to the dairy species (buffalo, cattle, sheep, goat, donkey, etc.) (7).

The portable milkmeter Lactocorder (WMB, Balgach, Switzerland) is normally used to record milk flow curve parameters. The latter have been well-studied in cattle (8–10), in sheep and goat (11) and, more recently, in buffalo (12–16). Briefly, the results are graphically represented in the screen of the milkmeter in three main different phases and, eventually, a fourth one. The first phase is the "milk let down phase" or lag time (LT), represented by the time elapsed between the attachment of the milking clusters and the time until there is a constant milk flow. The second one is called "plateau phase" (PPT), where the milk flow is constant. The third phase is the "decreasing phase" (DPT) that represents the time from the PPT until milk flow drops below 0.20 kg/min. An eventual fourth phase may be the "blind phase" (BT). The buffalo is often exposed to a long period of vacuum without any ejection of milk and sometimes a preliminary curve represents the cisternal fraction milk flow; this phase belongs to the LT. The BT (flow < 0.20 kg/min) occurs between the end of the DPT and the manual or automatic detachment of the milking group (both manual and automatic). Usually, the detachment is not performed promptly in order to collect the small amount of milk, the residual fraction, by stripping (often obtained by manual traction of the milking group by a milker) followed by a further overmilking before detachment of milking cluster.

Because of the udder anatomy and arrangement of the mammary tissue, cisternal fraction of milk and teat canal length are quite different in buffaloes compared with dairy cattle (13).

Milk flow curves are influenced by anatomical and physiological aspects (13, 17, 18), management (19, 20), and udder health status (12, 19, 21). Compared with bovines, buffaloes are characterized by a longer teat with a longer teat canal and by stronger muscular resistance of the teat wall. Therefore, a higher vacuum level is needed for opening the teat canal and starting milk ejection in this specie (7, 13, 18, 22). In buffalo routine management, the set-up of milking devices is a critical point and the characteristics of milking vacuum and milking pulsations are strictly connected with milk flow observations (18, 20, 22). It is well-established that udder health influences milk composition and milk flow parameters in dairy species, including buffalo (23, 24). In buffalo, as in other dairy species, the milk somatic cell count (SCC) is the most adopted indicator for udder health and mastitis (12, 23–25). Overall, in healthy conditions, buffaloes show lower milk SCC compared with bovines. The level of SCC is influenced mostly by environmental factors, in particular milking practices milking practices and farm hygiene. The aim of this study was to estimate the difference among milk flow curve types in terms of milk yield, SCC, and milkability traits in Mediterranean buffaloes in Italy.

MATERIALS AND METHODS

Animal and Herds

This study was carried out on 2,419 Mediterranean Italian water buffaloes from 187 herds of Latium Region (Central Italy). Data were collected from 2006 to 2019. Vacuum level recorded by a milking room vacuometer was set on average at 43.4 ± 4.2 kPa, ranging from 37.0 to 54.0 kPa. Among the 2,419 recorded milk flow curves, 131 (5.41%) were discarded as they did not reach the minimum flow necessary to return the main parameters. Therefore, 2,288 milk flow curves were available, of which 779 belonged to primiparous buffaloes with average days in milk (DIM) equal to 143, and 1,401 belonged to multiparous buffaloes with average DIM equal to 124. Milk yield per milking and milk flow traits were recorded with Lactocorder portable milkmeter (26). The pre-milking routine adopted was the standard, thus including teat cleaning, short massage, and subsequent drying. In all herds, the milking cluster was applied after pre-milking routine, whereas milking cluster detachment was manually removed even if automatic takeoffs were not present or not used; only in a few cases the use of automatic detachment was activated by the milker after the beginning of the alveolar milk emission.

Milk Flow Measurement Apparatus

The Lactocorder milkmeter (WMB, Balgach, Switzerland) starts recording information at the first teat cup attachment and finishes at the last teat cup detachment. The milkmeter was vertically attached to every milking place and inserted between the milking unit and milk pipeline by milk hose in the long milk tube. The measuring chamber consisted of one transmitting electrode and 60 vertically arranged electrodes. The evaluation of milk flow curve types was made by specific software available (LactoPro; WMB). The milk flow curve parameters and MY were recorded and a milk sample representative of the whole milking was automatically collected. For each buffalo, the device recorded 11 variables:

- MY (kg): total milk yield per head per milking from the beginning to the end of the mechanical milking;
- LT (min): lag time from the beginning of measurement until a 0.50 kg/min threshold in the milk flow was reached;
- MET (min): milk ejection time as time from milk flow rate >0.50 kg/min until milk flow decreases below 0.20 kg/min;
- PPT (min): plateau phase, as duration of plateau phase from the vertex of the incline phase to the vertex of the decline phase;
- DPT (min): time of decline phase, a period of milk flow from end of PPT to flow rate <0.20 kg/min at the end of milking;
- T400 (min): time to get the milk flow descending from 0.40 to 0.20 kg/min;

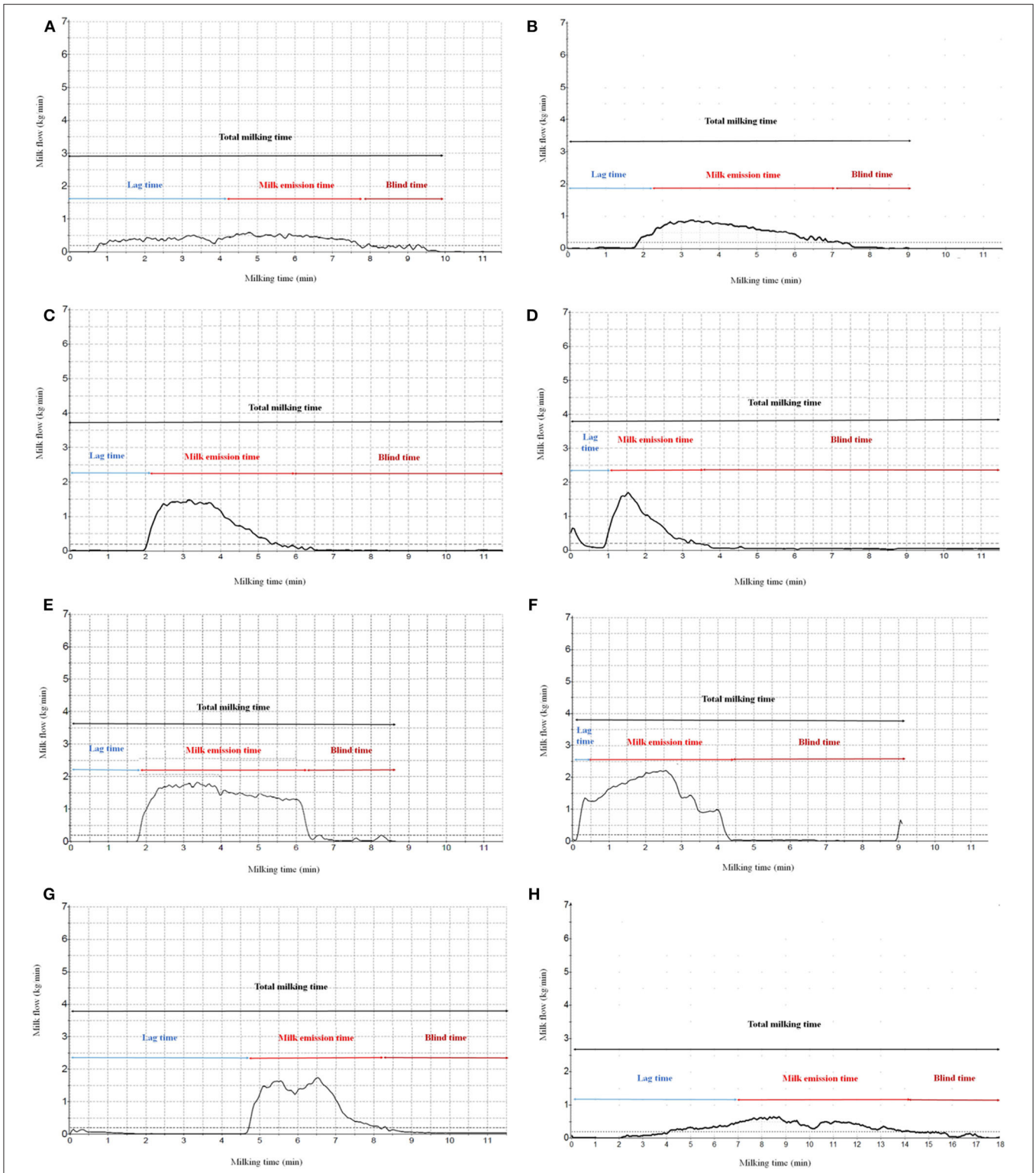

FIGURE 1 | Examples of **(A)** type 1 (very low milk flow and long milking time), **(B)** type 2 (very long and continuous decreasing phase time), **(C)** type 3 (similar time between plateau and decreasing phase), **(D)** type 4 ("triangular shape," high prevalence of bimodal curves), **(E)** type 5 ("rectangular shape," predominant plateau phase), **(F)** type 6 (predominant plateau phase and discontinuous decreasing phase), **(G)** type 7 (double-profile and evident disturbance of milk ejection during milking), and **(H)** type 8 (very long increase phase) milk flow curve.

- BT (min): time of mechanical overmilking, recorded if present at the end of DPT, with milk flow <0.20 kg/min;
- PFR (kg/min): peak flow rate in the main milking process within a time interval of eight measuring points measured within a time interval of 22.4 s;
- AFR (kg/min): average milk flow rate during milk ejection time;
- TMT (min): total milking time, that is, the time elapsed beginning the attachment of milking cluster at mammary gland and the detachment at end of milking, including stripping time and overmilking time after stripping if they are present;
- BIMO (%): bimodality of milk ejection, that is, delayed milk ejection at the start of milking (result of the interruption of the flow at the start of milking when cisternal milk is finished and alveolar milk is still not available) as a result of improper or absence of pre-stimulation of buffalo.

Milk Flow Curve Classification

The classification of curves was based on a visual inspection of shape of curve, MY, and milking flow parameters. Based on their appearance, the 2,288 milk flow curves were divided into 8 types (**Figure 1**) as:

- Type 1: with very low milk flow and long TMT;
- Type 2: with very long and continuous DPT;
- Type 3: with similar time between PPT and DPT;
- Type 4: triangular shape, with high prevalence of bimodal curves;
- Type 5: rectangular shape, with predominant PPT;
- Type 6: with predominant PPT and discontinuous DPT;
- Type 7: with double profile with evident disturbance of milk ejection during milking;
- Type 8: with a very long LT resulting from the slow release of oxytocin and higher resistance to opening of teat canals.

Somatic Cell Count Analysis

After collection, milk samples were refrigerated (5°C) and transferred to the milk laboratory of Istituto Zooprofilattico Sperimentale del Lazio e Toscana (Rome, Italy). The SCC determination was performed within 24 h from collection using a fluoro-opto-metric device (Fossomatic 5000 series; Foss Electric, Hillerød, Denmark). To achieve a normal distrbution of data, a logarithmic transformation was applied to SCC (27) and the somatic cell score (SCS, units) was obtained.

Statistical and Data Analysis

The effect of milk flow curve type was estimated on all milk flow parameters, MY, and SCS through one-way ANOVA in SPSS software (version 17.0; SPSS, Chicago, IL, USA). Differences in least-squares means were tested with Bonferroni *t*-test at $p < 0.05$.

RESULTS

Data Overview

The distribution of different types of milk flow curves is reported in **Table 1**. In both primiparous and ultiparous animals, the most represented curve was type 3 (**Table 1**), followed by type 6. The less represented one was type 1 (4.41%; **Table 1**). Milk flow curves with a gradual DPT (type 6) or continuous DPT (normal as type 2 or long as type 3) are represented in **Figure 1**. Triangular (type 4) and rectangular (type 5) shapes were characterized by short and long PPT, respectively, and longer and shorter DPT, respectively. Milk flow curves with a difficulty to milk release, resulting from an evident disturbance during mechanical milking, were classified in type 1 (long mechanical milking time), type 7 (double milk profile), and type 8. The trapezoidal shapes (types 2, 3, 6) are the most represented (59.53%), in comparison with rectangular shape or ideal curve (type 5, 10.62%) and the triangular shape (type 4, 9.48%). The curves with difficulties on milk emission (types 1, 7, 8) represented an important share (20.37%) of the total curves. The frequencies of the milk flow curve type were similar in primiparous and multiparous animals (**Table 1**).

TABLE 1 | Frequency of records (%) for the 8 types of curve defined.

Milk flow curve type	Whole dataset	Primiparous (n = 779)	Multiparous (n = 1,401)
1	4.41	5.01	4.14
2	14.42	13.48	14.49
3	27.32	30.42	25.70
4	9.48	8.99	9.85
5	10.62	10.01	10.78
6	17.79	17.20	18.70
7	6.08	5.13	6.28
8	9.88	9.76	10.06

Descriptive statistics of MY, SCS, and milk flow traits are presented in **Table 2**; MY averaged 3.95 ± 1.86 kg. In particular, primiparous and multiparous buffaloes showed an average MY of 3.66 ± 1.72 and 4.08 ± 1.89 kg, respectively. The total milking time was on average 11.29 min per milking; MET was on average 4.30 min, with a PPT:DPT ratio equal to 85.45%, whereas the average BT was 3.76 min. Means of PFR and AFR were 1.28 and 0.82 kg/min, respectively.

Patterns of Curve Types

Least-squares means (LSM) of MY ranged from 2.21 to 5.22 kg, estimated for curves in types 1 and 6, respectively. In the most frequent curve (type 3), LSM of MY was 3.29 kg. TMT ranged from 10.88 to 12.17 min, recorded for types 3 and 8, respectively. LT showed great variability, with maximum observed in type 1 (4.09 min) and minimum observed in type 6 (1.58 min). Minimum and maximum LSM of MET were 3.00 and 5.73 min, recorded for types 1 and 7, respectively. The most represented curve, type 3, showed LSM of LT and MET equal to 2.08 and 3.35 min. An opposite trend for PPT and DPT, calculated from PPT:DPT ratio, was observed in type 4 (17.86%) and type 5 (439.13%). T400 showed a maximum value in type 3 and represented on average 25.35% of the DPT (calculated between T400 and DPT ratio) and ranged from 17.85% (type 4) to 41.78% (type 1). BT ranged from 1.91 to 4.26 min, recorded in types 1 and 3, respectively. The BIMO was maximum (27.65%) in type 4 curves. Finally, LSM of SCS were from the highest (4.12 units) and the lowest (3.27 units) in types 6 and 1, respectively.

TABLE 2 | Overall mean (±standard deviation) and least-squares means (±standard error) of MY, milk yield per milking; BIMO, bimodality of milk ejection; PFR, peak flow rate; AFR, average milk flow rate; SCS, Somatic Cell Score; LT, lag time; MET, milk ejection time; PPT, time of plateau phase; DPT, time of decline phase; BT, time of blind phase; T400, time to get the milk flow from 0.40 to 0.20 kg/min; TMT, total milking time; in recorded curve types.

	MILK FLOW CURVE TYPES								
Parameter	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Overall mean**
MY, kg	2.21 ± 0.12^{f}	$3.53 \pm 0.10^{d,e}$	3.29 ± 0.05^{e}	$3.80 \pm 0.13^{c,d}$	$4.24 \pm 0.11^{b,c}$	5.22 ± 0.09^{a}	4.38 ± 0.17^{b}	4.43 ± 0.13^{b}	3.95 ± 1.86
BIMO, %	4.95 ± 2.17^{b}	10.61 ± 1.69^{b}	9.92 ± 1.20^{b}	27.65 ± 3.04^{a}	12.40 ± 2.12^{b}	10.07 ± 1.49^{b}	17.27 ± 3.22^{b}	12.78 ± 2.23^{b}	12.50 ± 33.70
PFR, kg/min	0.50 ± 0.04^{f}	1.10 ± 0.03^{d}	$1.29 \pm 0.02^{c,e}$	1.73 ± 0.05^{a}	$1.26 \pm 0.03^{c,e}$	1.47 ± 0.03^{b}	$1.15 \pm 0.04^{d,e}$	$1.18 \pm 0.04^{d,e}$	1.28 ± 0.57
AFR, kg/min	0.36 ± 0.03^{e}	0.63 ± 0.01^{d}	0.85 ± 0.01^{b}	0.96 ± 0.03^{a}	0.94 ± 0.02^{a}	0.98 ± 0.02^{a}	$0.68 \pm 0.02^{c,d}$	0.74 ± 0.02^{c}	0.82 ± 0.35
SCS, units	4.12 ± 0.18^{a}	$3.65 \pm 0.10^{a,b,c}$	$3.79 \pm 0.07^{a,b}$	$3.72 \pm 0.10^{a,b}$	$3.46 \pm 0.10^{b,c}$	3.27 ± 0.08^{c}	$3.63 \pm 0.15^{a,b,c}$	$3.70 \pm 0.11^{a,b}$	3.63 ± 1.67
LT, min	4.09 ± 0.42^{a}	2.13 ± 0.12^{c}	2.08 ± 0.09^{c}	$2.34 \pm 0.18^{b,c}$	$1.95 \pm 0.13^{c,d}$	1.58 ± 0.07^{d}	$2.31 \pm 0.17^{b,c}$	2.84 ± 0.15^{b}	2.19 ± 2.34
MET, min	3.00 ± 0.36^{d}	4.94 ± 0.15^{b}	3.35 ± 0.06^{d}	$3.50 \pm 0.12^{c,d}$	4.05 ± 0.10^{c}	$5.12 \pm 0.10^{a,b}$	5.73 ± 0.24^{a}	$5.24 \pm 0.18^{a,b}$	4.30 ± 2.33
PPT, min	1.24 ± 0.19^{c}	1.42 ± 0.06^{c}	1.41 ± 0.03^{c}	0.45 ± 0.03^{d}	3.03 ± 0.09^{a}	2.43 ± 0.07^{b}	2.31 ± 0.17^{b}	2.40 ± 0.11^{b}	1.82 ± 1.50
DPT, min	1.45 ± 0.26^{d}	3.27 ± 0.14^{a}	1.65 ± 0.04^{d}	$2.52 \pm 0.11^{c,b}$	0.69 ± 0.03^{e}	2.37 ± 0.07^{c}	$2.97 \pm 0.21^{a,b}$	2.34 ± 0.15^{c}	2.13 ± 1.90
BT, min	1.91 ± 0.26^{c}	$3.50 \pm 0.17^{c,e}$	4.41 ± 0.13^{a}	$4.26 \pm 0.23^{a,b,c}$	$4.16 \pm 0.22^{a,b,c,d}$	$3.48 \pm 0.14^{c,e}$	$2.87 \pm 0.24^{e,f}$	$3.27 \pm 0.22^{d,e}$	3.76 ± 3.19
T400, min	$0.60 \pm 0.13^{a,b}$	0.87 ± 0.07^{a}	0.48 ± 0.02^{b}	$0.45 \pm 0.03^{b,c}$	0.26 ± 0.02^{c}	0.53 ± 0.04^{b}	0.59 ± 0.07^{b}	0.54 ± 0.05^{b}	0.54 ± 0.79
TMT, min	$11.89 \pm 0.43^{a,b}$	$11.65 \pm 0.19^{a,b}$	10.88 ± 0.15^{b}	$11.28 \pm 0.27^{a,b}$	11.03 ± 0.25^{b}	11.02 ± 0.16^{b}	$11.78 \pm 0.28^{a,b}$	12.17 ± 0.25^{a}	11.29 ± 3.68

The fixed effect of milk flow curve type was significant at $p < 0.001$ for all traits.
$^{a-f}$Least squares means in a row without a common superscript letter differ ($p < 0.05$).

DISCUSSION

Milkability Traits of Mediterranean Italian Buffalo

Findings related to MY were similar to those reported in literature for buffaloes (12, 14, 15, 19, 25, 28); however, average SCS was higher if compared at the value of 3.49, found in a field study conducted on 225 Mediterranean Italian buffaloes (21).

The TMT of this study was similar to the average values (12.90 and 11.39 min, respectively, for different vacuum levels) observed in one farm with 450 buffaloes in Italy (20) but lower to the value (6.79 min without LT) reported for buffaloes present in eight herds of Northern Italy (14).

Overall, buffaloes of this study showed a long LT (2.19 min), that is, greater compared with values available in the literature (14, 15). The longer LT before milk ejection confirmed previous studies on Mediterranean Italian buffalo, where average LT was equal to 1.94 (14) and 2.42 min (20). In Murrah buffalo (29), different values have been related to different pre-stimulation milking routine and different feeding administration during milking, with LT ranging from 1.15 to 2.57 min.

LT is influenced by the anatomical characteristics of the mammary gland and teats, with regard to resistance of teat sphincters and release of oxytocin hormone. A correct udder pre-stimulation with the simultaneous addition of a concentrate integration in the milking room has positive effects on MY and milk flow (13, 29). In fact, 1 min of manual pre-stimulation produced the best results in terms of milk ejection, AFR, PFR, and TMT.

MET was about 38.09% of the total milking time, similar to the one reported in literature (15, 19). In this time, extraction of the maximum quantity of milk is obtained. Overmilking of BT was very long (33.03% of total milking time, on average) and similar to data reported in a study on 184 buffaloes (14). In the same study, BIMO was of control group and of buffaloes treated with oxytocin were 12.40 and 8.90%, respectively.

Average AFR and PFR were 0.82 and 1.28 kg/min, confirming findings available in literature (12, 14, 16, 19, 20), with small differences between morning and evening milking and in relation with the anatomical characteristics of teats (28).

The frequencies of curve types (**Table 2**) resembled findings on Jersey cows (30); in this study, about 66% of curves were rectangular and descending. In a field study carried out on 497 Holstein cows (31), 59.4% of total curves were those with a gradual or continuous decrease phase, similar to the percentage (59.53%) of the present study (type 2, type 3, and type 6). In the same study (31), only 6.4% of the curves were trapezoidal (it represents the optimal shape which includes a short and steep inclination phase, a plateau phase along a short and steep decline phase), whereas in the present study, type 5, similar in shape to the trapezoidal shape, was found on 10.42%, higher than the trapezoidal one. The same researchers reported that it may be useful to consider that the goal of most farmers is to select animals with milk emission similar to trapezoidal shape of milk flow curve, an indicator of a physiological letdown of milk.

In general, in this study, the BT phase was sometimes predominant when compared with other phases (**Figure 1**). Presence of long BT increases management costs and mastitis risk as a result of tissue stress and may indicate suboptimal udder pre-milking stimulation. In other species, it has been demonstrated that the reduction of BT is essential to improve udder health, longevity, and milking efficiency (30, 32, 33).

Fixed Effect of Milk Curve Type

According to the ANOVA (**Table 2**), LSM of MY in type 1 curve was the lowest. In fact, type 1 curve is characterized by very long TMT and thus limited MY, difficulties in LT resulting from changes in the anatomical parameters of teat canal and likely to low variation of oxytocin blood level. On the contrary, MY

of type 6 was the highest; this curve is characterized by a high PFR, long PPT, and probably by different milk distribution in one or more quarters (20). Types 2 and 3 curves (36.8% of total curves) were characterized by intermediate MY, with different lengths of DPT; this phase, where the milk flow is decreasing and continuous, is predominant with respect to PPT. At the udder level, the DPT mainly begins when milk flow from one or more quarters stopped. In contrast, at the quarter level, DPT starts when cisternal filling by alveolar milk is lower than intensity of milk removal (10). On the other hand, a short phase of decline is desirable to ensure the machine's adhesion time and milking stress within limits. A long decline is generally an indicator of an overmilking of one or more quarters. However, about 15–20% of curves for cattle were reported with a medium-long physiological DPT with a constant reduction of milk flow (26).

Type 4 curve is called "triangular shape" because it shows a very high PFR, short PPT, and long DPT; the BIMO was the highest in this type (**Table 2**). A similar type was found in high percentage (40% of total curves) in dairy dromedary camel (34), characterized by higher peak flow levels and short milking durations which depend mainly on the amount of milk stored in the udder.

Types 7 and 8 (15.95% of total curves) showed evident difficulty of milk emission with similar MY (4.38 vs. 4.43 kg, respectively; **Table 2**). In type 7, the double profile (**Figure 1**) is probably caused by an evident disturbance of milk ejection, related to blood oxytocin concentration changes, or to position changes of milking cluster during MET (15). Type 8 is characterized by a slight—but continuous—increase of milk ejection, probably as a result of a slow increase of blood oxytocin concentration and higher resistance to opening of teat sphincters.

Finally, the ideal milk emission type was recognized in type 5 curve, the "rectangular shape," with high PFR, AFR, and PPT:DPT ratio.

Regarding SCS, the most desirable value was found in curves of types 5 and 6; both SCC and SCS are closely related to the management of the farm, especially milking procedures (35, 36). In this paper, only data of a single farm were used and therefore the effect of type of management was minimized. In agreement with the literature (37), the greatest MY was found in correspondence of the lowest SCS (curves types 5 and 6) and vice versa; this could suggest that animals with optimal milk flow curves were characterized by less mammary gland epithelium stress and good milk flow and therefore showed favorable MY and SCS. On the other hand, it is established that suboptimal milk ejection patterns may be alert for udder infection and/or inflammation. However, the inverse trend of MY and SCS could be also partly caused by a dilution effect. In fact, in this study, there was no correction for the productivity level (genetic potential) of each buffalo. Genetically, high-producing heads are expected to be under oxidative stress, have greater susceptibility to udder issues, and overall present higher milk SCS.

CONCLUSION

The aim of this study was to estimate the differences among on milk yield, SCC, and milk flow traits recorded by a portable milkmeter in Mediterranean Italian buffaloes. We analyzed milk flow curves coming from more than 2000 Mediterranean Italian buffalo, and then we proposed a classification to highlight some significant difference among curves that could have an impact on milk production and udder health. Results highlighted a high prevalence of overmilking that may be responsible for the adoption of higher TMT in buffalo compared with cattle. In general, it is recommended to apply adequate udder stimulation before milking to reduce LT, increase AFR, and limit TMT. Moreover, adequate milking practices would translate into decrease of working time and improvement of farmer's income through better milk quality and less udder issues. The results of this study have allowed to identify optimal milk flow curve types in terms of milk production and udder health; such findings might be useful to buffalo breeders' associations to address farmers' management and also to define potential new breeding objectives. Further research is needed to investigate the variability of such phenotypes at the population level and understand if they may be exploited as indicator traits for breeding purposes.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because data that support the findings of this study are available at the corresponding author, [CB], upon reasonable request. Requests to access the datasets should be directed to carlo.boselli@izslt.it.

ETHICS STATEMENT

Ethical approval was not required for the present study, since we recorded only milking data during mechanical milking. Written informed consent for participation was not obtained from the farmers because collection and recording of data was carried out during official controls in milking parlour.

AUTHOR CONTRIBUTIONS

CB and AB designed and carried out the surveys in the milking parlour. CB processed the data. CB and AC discussed the results and wrote the draft. Finally, all authors revised the manuscript, contributed to the article and approved the submitted version.

FUNDING

This paper was based on the research project IZSLT 07/19 (G81F19000190001) funded by the Italian Ministry of Health (Rome, Italy).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 13 August 2020
doi: 10.3389/fvets.2020.00501

Establishment of a Stable β-Casein Protein-Secreted Laoshan Dairy Goat Mammary Epithelial Cell Line

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Mammary epithelial cells are widely used as models in mastitis research and as tools for mammalian bioreactors; however, the short lifespan of these cells limits their utility. Several mammal epithelial cell line models have been established; however, the secretion capacity and the bacterial sensitivity of these lines have not been effectively evaluated. In this study, a stable immortalized goat mammary epithelial cell (GMEC) line was constructed by transfection with the *SV40* gene. The monoclonal cells were then passaged through more than 50 generations after puromycin selection. The GMEC line was evaluated by reverse transcriptase polymerase chain reaction, the cell cycle, karyotype analysis, detection of apoptosis, Western blotting, and β-casein (CSN2) inducible assays. The GMEC line had a strong proliferation capacity relative to the primary GMECs. GMECs had the same karyotype as the primary cells. The GMEC lines maintained basic biological properties and had estrogen, prolactin, and progesterone receptors as same the primary cells. Additionally, the cells and the cell line could synthesize and secrete β-casein proteins. Finally, the rate of apoptosis of the transfected cells suggested that the cell line could provide a useful tool for signal research and mammary gland bioreactors.

Keywords: immortalization, goat mammary epithelial cells, SV40T antigen, β-casein secretion, cells apoptosis

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Edited by:
Domenico Vecchio,
Istituto Zooprofilattico Sperimentale del Mezzogiorno, Italy

Reviewed by:
Xuejun Gao,
Yangtze University, China
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Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: *26 April 2020*
Accepted: *02 July 2020*
Published: *13 August 2020*

Citation:
Zhang H, Liu T, Li B, Zhang K, Wang D, Liu Y, Ge L, Jiang Y and Su F (2020) Establishment of a Stable β-Casein Protein-Secreted Laoshan Dairy Goat Mammary Epithelial Cell Line. Front. Vet. Sci. 7:501. doi: 10.3389/fvets.2020.00501

INTRODUCTION

Mastitis is characterized by inflammation of the mammary glands induced by mechanical damage and microbial infection and can be classified as subclinical or clinical mastitis (1). In general, injury of the mammary glands results in bacteriological changes in milk and pathological changes in glandular tissues (2, 3). Subclinical mastitis in ewes is often induced by bacterial infections (4). Release of cytokines caused by both local and systemic inflammatory reactions also triggers an inflammatory cascade—the "acute phase response" during inflammation—and leads to the acute phase of coliform mastitis (5, 6). Recent work suggests that the effects of mastitis are not only restricted to the udder but also can affect reproductive efficiency (7). Thus, there is a pressing need to find ways to reduce the incidence of mastitis through the production of antimicrobial peptides. Previous study reported production of antimicrobial peptides in mammary tissue is an effective way to reduce incidence of mastitis (7). This methods usually employed transgenic methods, which generally involve developing a cell model for antibacterial and secretion assays. Therefore, a stable cell model is necessary for gene editing of animal reproductive traits and the use of the mammary gland as a bioreactor.

Mammary epithelial cells (MECs) play a vital role in the onset of the innate immune response caused by goat mastitis (8). Toll-like receptors (TLRs) are the first immune barrier in the mammary gland. TLRs that attach to the surface of cells can recognize specific patterns in pathogens and release cytokines, contributing to the recruitment and activation of effector immune cells in the mammary gland. TLRs also play a critical role in the identification of microbial pathogens (9, 10). Additionally, the milk produced and secreted from MECs is under the control of hormones after delivery. For these reasons, MECs are often target cells for mammary bioreactors (11). Therefore, the establishment of immortalized goat mammary epithelial cells (GMECs) is important for reducing the incidence of mastitis and the production of antibacterial peptides.

There are currently two methods for developing cell lines. The first is through the use of human telomerase reverse transcriptase (hTERT). For example, to construct MEC lines, Previous study isolated GMECs from the mammary tissue of dairy goats and immortalized them with human telomerase reverse transcriptase (hTERT) (12, 13). However, some studies have shown that immortalization of normal cells by transfected hTERT has low transfection efficiency and can induce dysplasia and even crisis (13). Furthermore, hTERT-expressing cell lines may experience crisis after prolonged lifespans, and immortality is often not a consequence of hTERT expression in normal diploid cells (12, 13). The second method for developing cell lines is through the SV40 method. For example, some studies have shown that the expression of SV40 large T antigen (*SV40 LT*) genes in healthy cells can circumvent the cell cycle M1 (senescence) through the inhibition of the p53 and p16 pathways, thereby resulting in tumorigenesis and immortalization of cells (14–16). The efficiency of *SV40 LT* gene infection is significantly higher than that of hTERT (17).

Here, purified MECs were isolated from goat mammary tissue, and a cell line was established through a transfer plasmid containing the *SV40 LT* gene, followed by evaluation of its bioactivity over 50 generations compared with primary cells. The assays showed that these cells preserved the key characteristics of MECs and thus provide a robust model for mammary secretion assays and disease research.

MATERIALS AND METHODS

Ethics Statement

The 2-year-old (45 days post-parturition) Laoshan dairy goats used in this study were bought from Zhengda Company from China (Taian, China) and accommodated in appropriate livestock housing and fed ad libitum. Goats were sacrificed with sodium barbital after anesthesia. All procedures involving animals were approved by the Animal Care and Use Committee of Shandong Agricultural University. Mammary tissue was collected after the goat was injected with sodium barbital (5 mg/kg) following anesthesia (subcutaneous injection).

Reagents and Chemicals

DMEM/F-12 and FBS were purchased from BI (FBS, BI, Kibbutz, Israel). Insulin, hydrocortisone, and collagenase I were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin was purchased from Gibco (Gibco Lab., Grand Island, NY, USA), and ovine prolactin was purchased from SGMEC (Israel). Rabbit anti-keratin 18 (CK18), rabbit anti-progesterone receptor (PR), rabbit anti-estrogen receptor α (ERα), rabbit anti-prolactin receptor (PRLR), rabbit anti-β-casein (CSN2), and rabbit anti-cleaved caspase-3 were obtained from Abcam (Cambridge, UK).

Isolation and Purification of Primary GMECs

MECs were derived from a mastitis-free Laoshan dairy goat that was in its lactation phase. Mammary tissue was obtained by a surgical operation after anesthesia by chloral hydrate. The tissue was then disinfected and cut into 1-mm^3 cubes. The 1-mm^3 tissues were then digested with 0.2% collagenase I at 37°C for 4 h, filtered through a 200-μm mesh screen and washed with phosphate-buffered saline (PBS) until the supernatant was free of turbidity. The cells were seeded into DMEM/F12 medium and incubated in an incubator at 37°C with 5% CO_2. Then the cells were purified with differential digestion methods.

Transfection of Cells With the *SV40 LT* Gene

The packaged virus which containing *SV40 LT* gene were obtained and concentrated by human 293T cells. In details, Cell supernatants were then harvested after co-transfection with pLVX-EGFP-T2A-Puro-SV40T (10 μg), psPAX2 (5 μg), and pCMV-VSV-G (7.5 μg) into human 293T cells (6×10^6 cells/100-mm dish) for 48 h using the TransIntro^{TM} EL transfection reagent per the manufacturer's instructions. The cell supernatant medium was then filtered through a 0.45-μm membrane filter and mixed with 60% 5 × PEG8000 at 4°C overnight. The supernatant was discarded after centrifugation at 4,000 g for 20 min, and the tube was left to settle for 2 min. Finally, the residual liquid was removed, and an appropriate amount of

TABLE 1 | Primer pairs used in this study.

Prime name	Primer sequence	Reactive condition PCR	Length/bp
SV40 LT	F: AGTGGCTGGGCTG TTCTTTT	95°C for 5 min; s 94°C 30 s, 60°C 30 s	671
	R: ATGGGAGCAGTG GTGGAATG	72°C 30 s, 34 cycle; 72°C 10 min	
ACACA	F: GGTCAGCTCAGA CACGCTCT	95°C for 5 min; s 94°C 30 s, 55°C 30 s	838
	R: GTTAGGGAAGT CATCCGCGT	72°C 30 s, 34 cycle; 72°C 10 min	
CSN2	F: ACAGCCTCCCA CAAAACATCC	95°C for 5 min; s 94°C 30 s, 52°C 30 s	300
	R: TGAGAAAGGG ACAGCCACGGA	72°C 30 s, 34 cycle; 72°C 10 min	
GAPDH	F: AAGTTCCACGGCA CAGTCA	95°C for 5 min; s 94°C 30 s, 56°C 30 s	473
	R: GGTTCACGCCCA TCACAA	72°C 30 s, 34 cycle; 72°C 10 min	

lentivirus solution was added to dissolve the lentivirus virus. The purified lentivirus was used for the GMEC transfection procedures in the presence of 0.5 μg/ml of polybrene. Isolated primary GMECs were cultured in 100-mm culture-grade plastic dishes for 12 h before transfection with the virus that containing *SV40* LT gene. Then the virus were added into cells medium for cells infection. After 4 days of incubation, the cells were cultured with growth medium containing 1 μg/ml puromycin to select resistant cells.

Cell Cycle and Growth Curve of GMECs

The cell cycle was evaluated by flow cytometry. Primary GMEC and GMEC lines (50 passages) were harvested and washed twice with cold PBS, followed by resuspension in cold 75% ethanol and fixation overnight at 4°C. The cells were then stained with PI/RNase Staining Buffer (BD, USA) per the manufacturer's protocol after washing cells twice with cold PBS. Finally, the aforementioned cells were detected using a BD FACSCalibur™ flow cytometer. Primary GMEC and GMEC lines were seeded in 96-well-plates (cell number 1,000/well) for 7 days. The number of cells was counted every day.

Cell Immunofluorescence

The GMEC line and primary GMECs were seeded on slides of cells. GMECs were rinsed with PBS, fixed by cell immunofluorescent fixative, permeated with 0.5% Triton X-100 and blocked with 5% FBS. Primary rabbit anti-CK18 (1:100), anti-ERα (1:100), anti-PR (1:100), and anti-PRLR (1:200) were incubated with cells overnight at 4°C. The coverslips were then washed three times with PBS. Alexa Fluor 555-labeled donkey anti-rabbit IgG secondary antibodies (H+L) (1:500) were added to the coverslips and incubated for 50 min in the dark. The coverslips were washed three times with PBS. Hoechst 33342 was added to the coverslips and incubated for 15 min at 37°C. The coverslips were again washed three times with PBS. Finally, the stained cells were visualized with confocal microscopy.

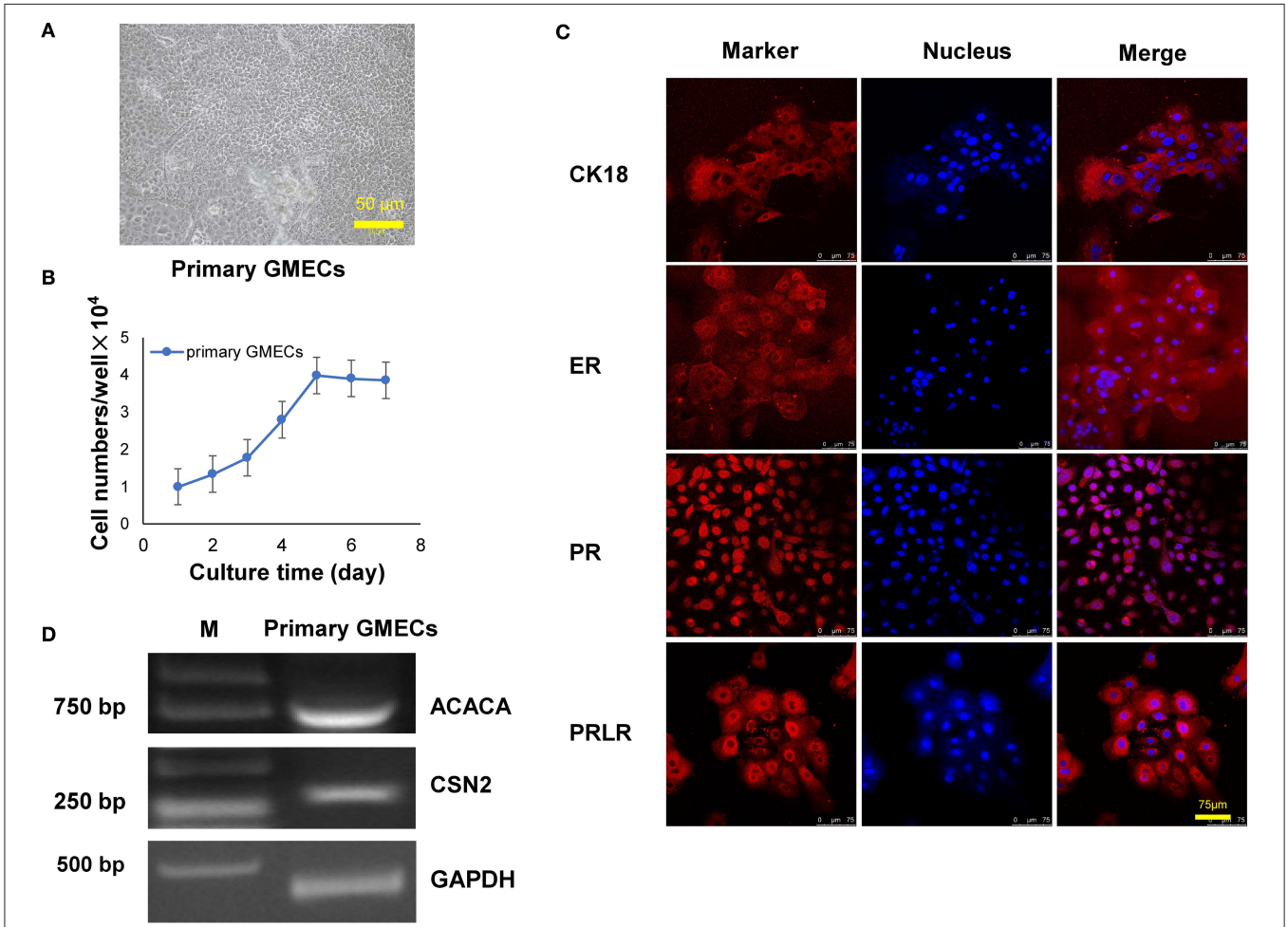

FIGURE 1 | Characteristics of primary goat mammary epithelial cells (GMECs). **(A)** Primary GMECs observed under a light microscope (100×). **(B)** Growth curve of primary GMECs that was constructed from cell counts made on different days. **(C)** The immunofluorescence assays of primary GMECs for cytokeratin CK18, ERα, PR, and PRLR. **(D)** RT-PCR analysis of *ACACA* and *CSN2* in primary GMECs.

Karyotype Analysis

Primary GMECs and GMEC-line (60 passages) were incubated into DMEM/F12 medium with 10% FBS for 12 h. Colchicine (1 μg/ml) was then added to the cells 5 h after the cells were overfilled. Next, the cells were digested and resuspended in 10 mL of 0.075 M KCl and incubated at 37°C for 25 min. The pre-cooled fixing solution (methanol:glacial acetic acid, 1:3, v/v) was added for pre-fixation. Finally, cells were dropped onto ice-cold glass slides and stained with PI for 10 min. The cells were examined by confocal microscopy after washing with PBS three times.

RT-PCR Assays

Primary GMECs and GMEC-line cells (50 passages) were incubated in DMEM/F12 medium for 12 h. Total RNA was extracted using the TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using the PrimeScript^{TM} RT Reagent Kit per the manufacturer's protocol. Finally, PCR amplification of the genes of interest was performed. The amplification primers of the *GAPDH*, *ACACA*, and *CSN2* genes were designed following the general PCR primer design principle and were synthesized by Sangon Biotech (Shanghai) Co., Ltd. PCR amplification was conducted in 25-μL reaction systems (**Table 1**).

Inducible Expression of CSN2 in GMECs

GMECs and goat fibroblasts were incubated in DMEM/F12 medium with 10% FBS for 12 h. The cell medium was then changed with serum-free induced medium (DMEM/F-12, 5 μg/mL insulin, 5 μg/mL prolactin, 10 ng/ml Epidermal Growth Factor (EGF) and 1 μg /mL hydrocortisone after the cells reached 80% confluency). The cell medium was then harvested after the cells had been induced for 48 h. The supernatant was collected after freeze-drying for 12 h. The proteins were then detected with the SDS-PAGE assay.

Western Blotting

Primary GMECs and GMEC-line cells were collected and lysed in RIPA buffer containing PMSF (1 mM) at 4°C for 30 min, followed by centrifugation at 12,000 g for 10 min to collect the supernatant. Proteins were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked

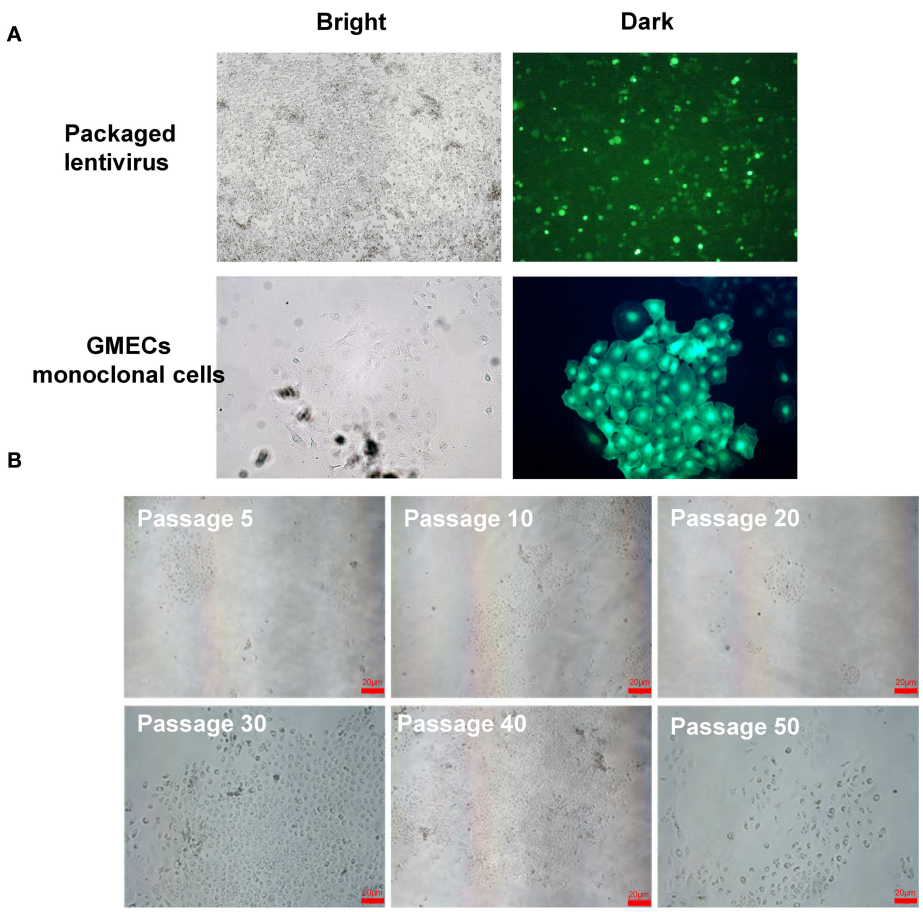

FIGURE 2 | Lentivirus packaging and monoclonal selection of GMECs. **(A)** The process of packaging lentiviral particles of plasmids containing the *SV40 LT* gene. Image of 293T cells with the transfer plasmid containing the *SV40* gene (top). Monoclonal GMECs obtained after puromycin selection observed through a microscope (bottom). **(B)** Cellular morphology of the GMEC line at 5, 10, 20, 30, 40, and 50 passages. No morphological differences were observed.

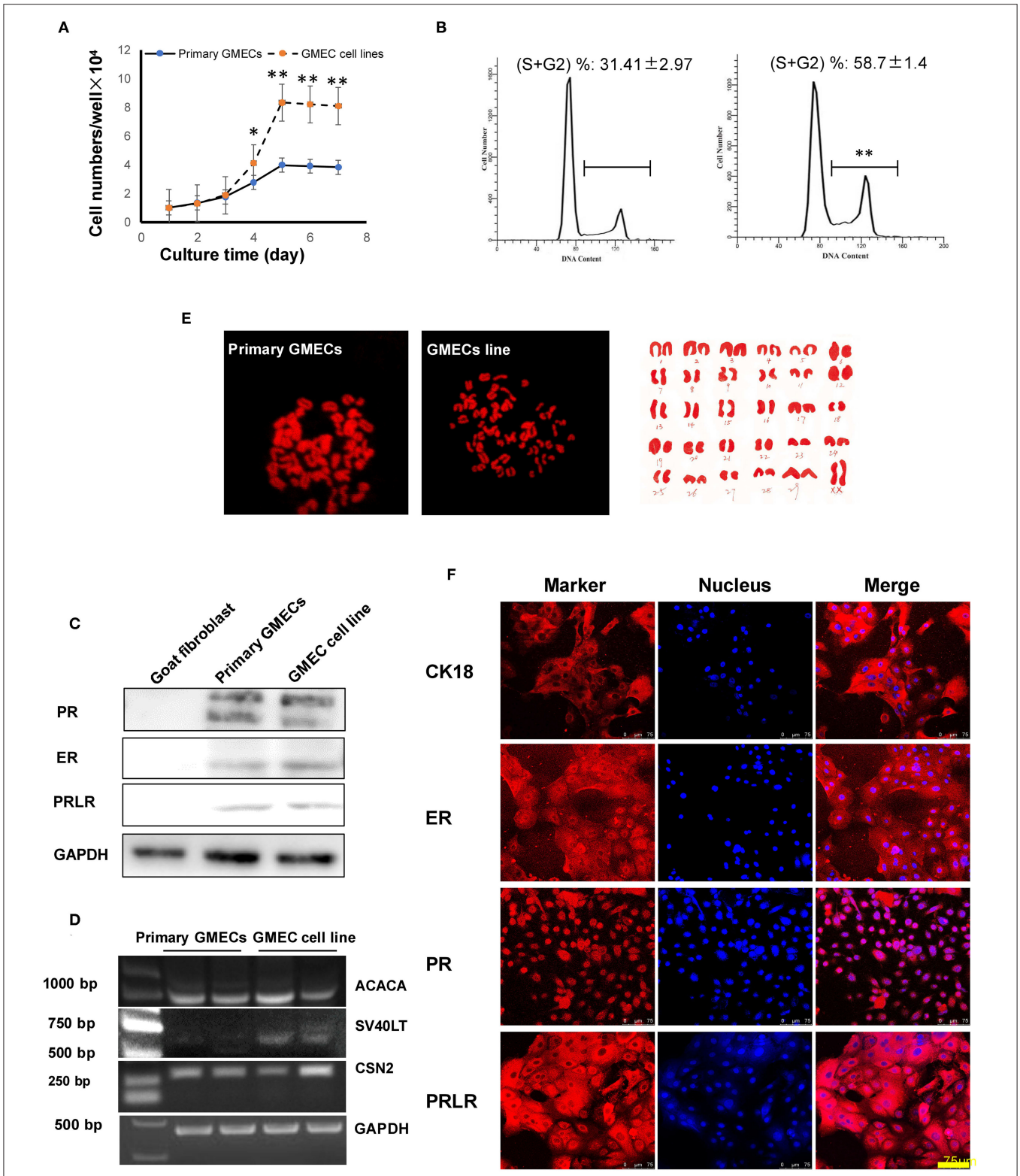

FIGURE 3 | Characteristics of the GMEC line. **(A)** Comparison of the proliferation ability between the GMEC line and primary GMECs. (*$p < 0.05$). **(B)** Comparison of the cell cycle between primary GMECs and the GMEC line. **(C)** Western blotting analysis of ERα, PR, and PRLR. Expression of ERα, PR, and PRLR proteins was observed in both primary GMECs and the GMEC line. **(D)** RT-PCR assays of *SV40T CSN2* and *ACACA* mRNA. *SV40T* was detected in the GMEC line but not in primary GMECs. **(E)** Chromosome counts were evaluated by karyotype analysis in the GMEC line. **(F)** Immunofluorescence images of the GMEC line detected by cytokeratin CK18, ERα, PR, and PRLR, confirming its mammary epithelial attributes.

with 5% skim milk powder for 90 min and incubated with anti-ERα (1:100, Abcam), anti-PR (1:1,000, Abcam), anti-PRLR (1:300, Abcam), anti-cleaved caspase 3 (1:1,000, Abcam), anti-CSN2 (1:1,000, Abcam), and anti-GAPDH (1:10,000, Abcam) polyclonal antibodies overnight at 4°C. After three washes with TBST, the membrane was incubated with goat anti-rabbit IgG (H&L) secondary antibodies for 50 min at room temperature, followed by another three washes with TBST. Finally, the membrane was covered with BeyoECL Moon ECL Kit (Beyotime), and its chemiluminescence was detected using a chemiluminescent detector.

Cell Apoptosis Assays

GMEC-line cells were collected after infection with lipopolysaccharide (LPS) for 48 h. The cells were then harvested and stained with a BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit per the manufacturer's protocol. The cells were detected with flow cytometry.

Statistical Analysis

Data from RT-PCR, cell proliferation analysis, and cell cycle, and CFU count assays were analyzed using SPASS software (SPSS, Chicago, IL, USA). Fold changes in target gene expression were presented as means ± SEM and were compared using one-way ANOVAs followed by Newman–Keuls tests. $P < 0.05$ were considered statistically significant.

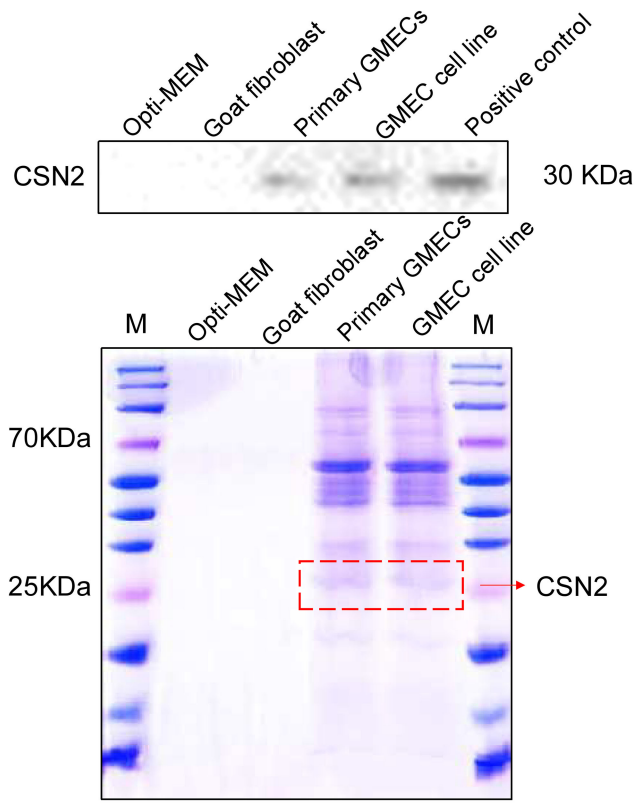

FIGURE 4 | Inducible expression of casein protein. SDS-PAGE and Western blotting analysis of the CSN2 protein. Inducible expression of CSN2 detected by SDS-PAGE in primary GMECs and the GMEC line. Western blotting analysis of inducible-expressed CSN2 protein.

RESULTS

Isolation and Identification of Primary GMECs

Primary GMECs were isolated from mammary gland tissue and seeded into Petri dishes. **Figure 1A** shows the purified MECs. GMECs grew in the medium, and the shape of the cells resembled paving stones (**Figure 1A**). Cell proliferation capacity was then assessed by its growth curve. Primary cells exhibited a steady pattern of growth similar to normal cells (**Figure 1B**). Cell markers were detected by immunofluorescence assays and the red signal exhibited by CK18, ER, PR, and PRLR for antibody-positive cells (**Figure 1C**). Primary cells were then assessed by RT-PCR assays. The band in **Figure 1D** indicated that the cells could express the *CSN2* and *ACACA* genes.

Establishment of the GMEC Line

The GMEC line was obtained by a transfer plasmid containing *SV40 LT* genes. Purified high-titer lentiviral particles were obtained after co-transfection with pLVX-EGFP-T2APuro-SV40 vector and pCMV-VSV-G and psMAX2 vectors in 293 T cells (**Figure 2A**). Monoclonal GMECs were selected by puromycin and were obtained after co-incubation with high lentiviral particles for 7 days (**Figure 2A**). Cells were then obtained from a single clone and subsequent passage from one generation to more than 60 passages. The shape of the cells resembled paving stones similar to the primary cells in different passages (**Figure 2B**). The green fluorescence of the GMEC-line cells ultimately disappeared.

The GMEC Line Sustained Its Biological Characteristics

The GMEC line proliferation capacity was evaluated. The proliferation rate of the GMEC line increased significantly compared with primary GMECs after 4 days ($p < 0.05$) (**Figure 3A**). The cell cycle assay showed that the GMEC line had longer G1 and S phases compared with the primary GMECs ($p < 0.05$; **Figure 3B**). The bioactivity of the GMEC line was then assessed by Western blotting and RT-PCR. The Western blotting assay showed that the cell line retained PR, ERα, and PRLR similar to primary GMECs (**Figure 3C**). RT-PCR assays indicated that the cells could express *ACACA* and *CSN2* mRNA. However, *SV40 LT* mRNA was only detected in the GMEC line and not in primary cells (**Figure 3D**). The karyotypes of the cell lines were then evaluated. Both the primary cells and the GMEC lines had 30 pairs of chromosomes ($2n = 60$; **Figure 3E**). Finally, cell line markers were assessed by immunofluorescence assays. The red image shown in **Figure 3F** shows that the cell line retained CK-18, ERα, PR, and PRLR.

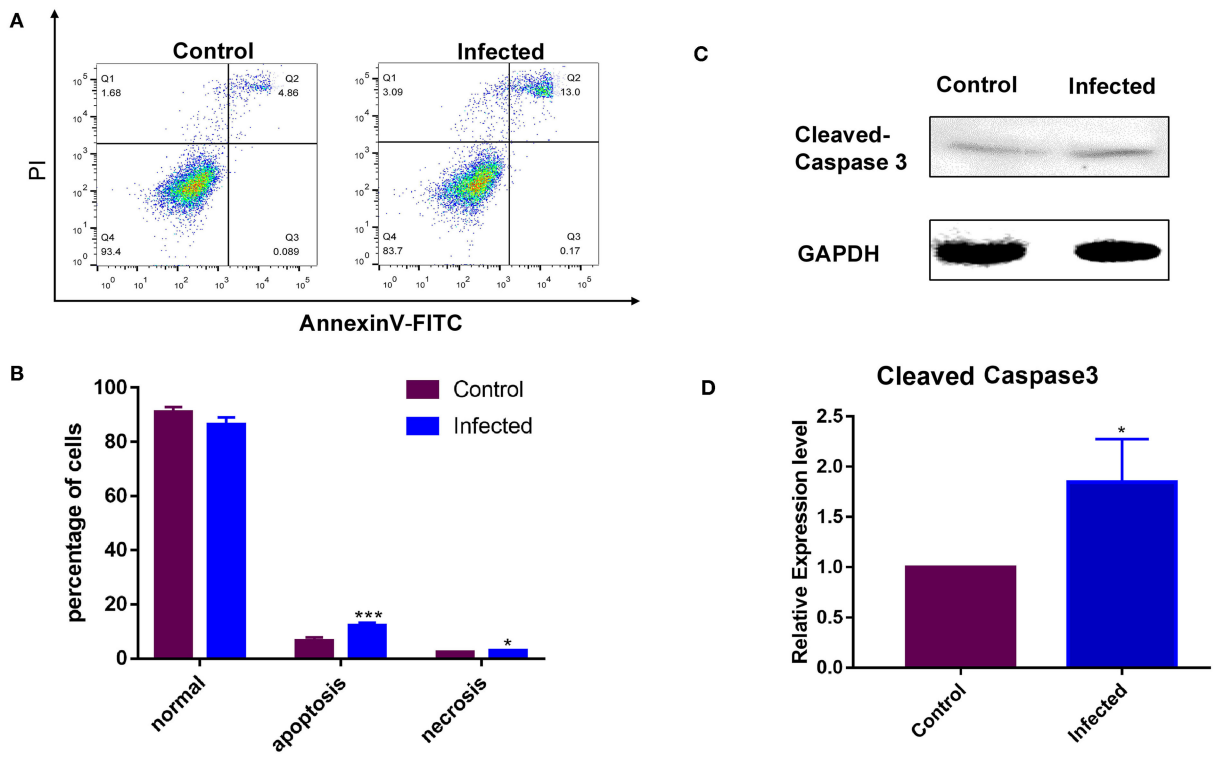

FIGURE 5 | Susceptibility analysis of the GMEC-line cells activated by LPS. **(A)** Rate of cell apoptosis in normal GMEC-line and LPS-infected GMEC-line cells. **(B)** Significant differences were observed between normal and apoptotic cells after infection (*$p < 0.05$). **(C)** Western blotting analysis of cleaved caspase-3. **(D)** Significant differences were observed between normal and apoptotic cells after LPS stimulation (**$p < 0.01$).

Secretion of CSN2 Protein in the GMEC Line

The secretion properties of cells were assessed by their inducible expression capacity. Secretion of the CSN2 protein was tested by SDS-PAGE and Western blotting analysis. The expression of CSN2 and other proteins is shown in **Figure 4**. The expression of these proteins was not detected in the goat fibroblasts cells and the negative control (**Figure 4**).

The GMEC Line Is Sensitive to LPS

The rate of apoptosis was used to estimate the susceptibility of the cell line to LPS. Non-infected GMEC line as a control group. A Western blotting analysis was conducted following incubation with LPS for 24 h. The LPS sensitivity of the GMEC line was estimated as the rate of apoptosis of GMECs. The rate of apoptosis of GMEC lines was much higher than that of the control ($p < 0.001$; **Figures 5A,B**). The expression of cleaved caspase 3 protein in GMEC lines was stronger than that of the control ($p < 0.01$; **Figures 5C,D**).

DISCUSSION

Mastitis is an important disease that causes substantial losses of dairy goats in the goat industry. Specifically, mastitis results in reductions in milk production and reproductive failure (18, 19). Generally, treatments with antibiotics provide an important method for preventing the occurrence of mastitis; however, this approach generates much environmental pollution (20). The development of gene-editing and transgenic strategies provides alternative, more environmentally friendly approaches for reducing the morbidity of mastitis. Furthermore, mammary bioreactors provide an effective means for building a protective barrier to resist bacterial infection, especially during the secretion phase (11). Thus, the establishment of a MEC line with stable secretion properties is important for mastitis research and the development of mastitis-resistant dairy goats. Here, we built a GMEC line that retained the bioactivity of MECs. These cells also retained their capacity for secretion and their sensitivity to LPS, confirming that this cell line could be used as a future tool for breast bioreactors and mastitis research.

Primary GMECs can be obtained via two methods: the tissue-block method or by digestion. Highly purified GMECs are more easily obtained by digestion relative to the tissue-block method. In this study, primary purified GMECs were taken from a Laoshan dairy goat by digesting mammary gland tissue pieces with collagenase I. This method differs from that of Shi et al. (21), as they obtained MECs by cultured tissue blocks. However, their method required more than 6 days; in addition, the purity of the cells was lower than that obtained by the digestion method (22, 23). Cell bioactivity was assessed by CK18, ER, PR, and PRLR proteins. Previous studies have reported that CK18 is an epithelial cell-specific protein marker; thus, the positive images

of CK18 in GMECs indicated that the cells that we obtained were, in fact, epithelial cells (21). We also detected the expression of ER, PR, and PRLR in primary GMECs, which is consistent with a previous study (24). Previous studies have reported that the estrogen, progesterone, and prolactin receptors are crucial for mammary development. The prolactin receptor is especially important, as a previous study suggested that prolactin could promote mammary development via activation of the Jak2/Stat pathway (25). The *ACACA* and *CSN2* genes are also recognized as important for controlling the contents of sheep milk. Both of these genes were evaluated by RT-PCR, and mRNAs in the cell lines indicated that GMECs retained the proteins encoded by these transcripts as has been previously shown (24). The aforementioned evidence indicates that these monolayer cells retain their functional proteins and that the cell line consisted of MECs.

The lentiviral system provides an efficient means for transfection to mediate the infection of non-dividing cells. This system also permits large genetic payloads and maintains stable long-term transgene expression (26). The *SV40 LT* could induce the immortalization of cells by promoting the bypassing of the M1 phase of the cell cycle (senescence) (16), it is also binding the retinoblastoma protein (Rb) and the tumor suppressor protein p53 and finally inhibition cell lines transform into primary one. Potential tumorigenesis of SV40 LT mainly reflected in the binding ability to p53, In other words, although the tumor generation probability is very low, SV40 LT gene has potential capacity causing tumorigenesis (16, 26). In this study, the *SV40 LT* gene was packaged into a lentivirus system by a three-plasmid system and infected into primary GMECs. After selection by puromycin, the monoclonal cells were successfully obtained and amplified. Loss of GFP expression in cell lines suggested that the GFP mRNA was degraded in the cytoplasm, consistent with the findings of a previous study (18).

The bioactivity of the cell line was evaluated, and the cell line retained its proliferation capacity in addition to the expression of CK18, ER, PR, and PRLR as in primary MECs. The expression of ERα, PR, and PRLR was also demonstrated by the Western blotting analysis. Moreover, the *ACACA* and *CSN2* genes were expressed in the GMEC line as well as in primary GMECs; however, the *SV40 LT* gene was only expressed in the GMEC line. Karyotypic characterization also demonstrated the stability of the GMECs. Thirty pairs of chromosomes were detected from the GMEC line in contrast to the number of pairs of chromosomes that has been reported by previous studies. For example, *SV40 LT*-transfected cells have been reported to have chromosomal abnormalities, such as chromosomal duplications (27). However, the karyotype analysis in our current study indicated that our GMEC line had normal numbers of chromosomes.

MECs are the cells involved in lipid synthesis, storage, and milk secretion, and their secretion ability is controlled by a series of hormones, such as prolactin and insulin (28). CSN2 is the main protein secreted from the breast and has a molecular weight between 20 to 30 kDa (29, 30). In our study, CSN2 was detected in the primary GMECs and cell lines similar to previous studies. The application of prolactin and insulin is key for CSN2 secretion. In previous assays, Wang et al. (31) reported that prolactin was necessary for casein synthesis in the lactating mammary glands of goats. Another study showed that insulin can stimulate and regulate milk protein synthesis in GMECs (32) and can also play an essential role in the accumulation of mRNA in mouse MECs (31). Our results indicated that both prolactin and insulin are essential for CSN2 secretion.

The susceptibility of GMECs is important for the construction of cell models. LPS was used as an inducer in this study for its immunological stimulation and toxic pathophysiology. Some studies have indicated that primary GMECs are often activated by LPS and used as research models for mastitis (33). For this reason, the reaction of the cell line was evaluated after treatment with LPS. Activation of the TLR4 signaling cascade by LPS promoted the transcription of inflammatory cytokines, which eventually led to cell apoptosis and death (34). Caspase-3 plays a key role in the fragmentation of DNA and inducing morphological changes associated with apoptosis (35). In our assay, cleaved caspase-3 was significantly increased after the GMEC line was treated with LPS, indicating that the GMEC line was susceptible to LPS.

CONCLUSIONS

In sum, a MEC line from Laoshan dairy goats was established via a transfer plasmid containing the *SV40 LT* gene. The bioactivity of the GMEC line was evaluated, and the cells retained ER, PRLR, and PR proteins; the cells also retained their capacity for secretion. The LPS stimulation assay indicated that the cell line provided a robust model for mastitis research and that it could also be used for mammary gland bioreactors.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of Shandong Agricultural University.

AUTHOR CONTRIBUTIONS

YJ, HZ, and FS designed the experiments and drafted the manuscript. HZ, TL, KZ, DW, and YL carried out the animal care, sample collection, and performed the experiments. BL and HZ performed the data processing and biological information analysis. FS and LG interpreted the data. HZ and FS conceived the study and wrote the manuscript. All authors read and approved the final draft.

FUNDING

Financial support for this research was provided by two grants from the Shandong Natural Science Foundation (Grant Number: ZR2016CQ25) and Shandong Agricultural Breed Project, 2019LZGC019.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 18 August 2020
doi: 10.3389/fvets.2020.00464

Reducing Ruminal Ammonia Production With Improvement in Feed Utilization Efficiency and Performance of Murrah Buffalo (*Bubalus bubalis*) Through Dietary Supplementation of Plant-Based Feed Additive Blend

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *28 April 2020*
Accepted: *23 June 2020*
Published: *18 August 2020*

Citation:
Chanu YM, Paul SS, Dey A and Dahiya SS (2020) Reducing Ruminal Ammonia Production With Improvement in Feed Utilization Efficiency and Performance of Murrah Buffalo (Bubalus bubalis) Through Dietary Supplementation of Plant-Based Feed Additive Blend. Front. Vet. Sci. 7:464. doi: 10.3389/fvets.2020.00464

The study evaluated the potential of blends of eucalyptus oil and aqueous extract of mulethi (root of *Glycyrrhiza glabra*) to reduce rate of ruminal ammonia production without affecting feed digestion to improve nitrogen utilization efficiency and performance of Murrah buffalo (*Bubalus bubalis*). Based on preliminary independent studies with graded doses of eucalyptus oil and mulethi root aqueous extract in modulating *in vitro* rumen fermentation, four blends of feed additive comprising graded doses (5, 10, 15, and 25 μL) of eucalyptus oil and a fixed quantity (15 μL) of aqueous extract of mulethi roots were prepared and examined for their effects on *in vitro* rumen fermentation and on methane and gas production in 100-mL calibrated glass syringes by standard IVGP protocol. Rumen liquor was collected from four rumen fistulated Murrah buffaloes fed a total mixed ration. Out of four blends, blend-1 comprising 5 μL of eucalyptus oil and 15 μL of aqueous extract (233.6 g/L DW) of mulethi per 40 mL of *in vitro* medium was found to reduce ammonia production significantly ($p < 0.001$) without affecting feed digestibility. An equivalent dose of blend-1 (10.5 mL of eucalyptus oil and 7.35 g of mulethi root powder/h/day) fed to four rumen fistulated buffaloes for 24 days resulted in 50% reduction ($p < 0.05$) in rumen ammonia level with no inhibition in feed fermentation or short-chain fatty acid production. The total bacterial population including *Ruminococcus albus*, *Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens*, and *Megasphaera elsdenii* as well as anaerobic fungi and methanogenic archaea remained unaffected ($p > 0.05$). Twelve buffalo calves (avg. BW 137.5 ± 9.2 kg, 8–12 months old) divided into two groups of six each and fed a total mixed ration (concentrate: roughage; 60:40) with or without supplementation of blend-1 for about 3 months demonstrated 14% increase ($p < 0.05$) in average daily gain in BW with a trend ($p < 0.10$) in improvement of feed or protein utilization efficiency (1.4 vs. 1.1 g crude protein/g average daily gain; 21.4% increase). Thus, supplementation of eucalyptus oil–mulethi root blend could reduce ruminal ammonia production and improve feed utilization efficiency in ruminants.

Keywords: eucalyptus oil, licorice, nitrogen efficiency, plant bioactives, ruminal ammonia production

INTRODUCTION

While livestock production forms one of the pillars of the global food industry, there have been huge societal concerns over the high contribution from livestock farming to atmosphere polluting gases such as methane (CH_4), nitrous oxide (N_2O), and ammonia (NH_3). Methane and nitrous oxide are greenhouse gases (GHGs) that contribute to climate change, while ammonia is a potent environmental pollutant and liable for depletion of oxygen in water bodies, reduction of soil pH, and generation of harmful aerosol fine particles (PM2.5) and accompanied with an augmented threat of pulmonary diseases (1). N_2O, the most subtle GHG, has 296 times more global warming potential than CO_2 (2) and is also responsible for ozone depletion. Nitrogen present in animal waste is directly converted to N_2O via microbial nitrification and denitrification, or indirectly via volatilization of ammonia gas followed by atmospheric oxidation of ammonia to N_2O (3).

The efficiency of nitrogen utilization in ruminants is very low due to deamination of most of the amino acid nitrogen entering the rumen, resulting in a high rate of ammonia production exceeding the capacity of rumen microorganisms for utilization of ammonia (4, 5). Animal nutritionists have attempted to combat excess ruminal nitrogen degradation using naturally insoluble proteins or reducing solubility artificially by heat and formaldehyde treatments. However, a substantial increase in feed cost and potential hazards of residual formaldehyde for human health limit their application (6, 7). Monensin, an ionophore antibiotic, also reduces the ruminal degradation of amino acids (8), but inclusion of antibiotics in animal rations is worrisome. Therefore, increasing interest in use of plant bioactive compounds such as polyphenols, saponins, and essential oils in modulating rumen fermentation and protein degradation is based on their potent antimicrobial properties and safety in animal products (9). Essential oils are reported to inhibit the breakdown of proteins in the rumen, thus increasing the availability of dietary proteins to the ruminants (10, 11). Plants containing saponins have been demonstrated to shift rumen microbiome to increase efficiency of microbial protein synthesis (12, 13); however, these effects are dependent on the types of saponins as well as diets of the animals (14, 15). The major component of eucalyptus essential oils has been described as 1,8-cineole (eucalyptol), besides cryptone, α-pinene, and others (16). Mulethi (*Glycyrrhiza glabra* L.) root contains broad-spectrum bioactive compounds, i.e., flavonoids (liquiritigenin, iso-liquiritigenin), iso-flavonoids (formononetin, biochanin A), terpenoids (glycyrrhetic acid, glycyrrhizin), phenolic acids, and others, which were demonstrated to have anticancer and antioxidant properties (17, 18).

Traditionally portrayed as "black gold," buffaloes are favorite multipurpose animals of farmers and are in fact the "bank on hooves" with a huge potential for social and economic changes for the agrarian community in Asian countries (19). Buffalo have been an integral part of livestock agriculture in Asia for more than 5,000 years, producing milk, meat, hides, and draft power. India is home to about 57% of the world's buffalo population (20) and during the past 70 years, the contribution of buffalo of +50% in the milk pool elevated India to the no. 1 world position in total milk production, while buffalo meat export earned India another distinction of being the largest beef (buffalo meat) exporting country in the world (21). Scanty *in vitro* research studies proposed reducing ammonia emission from the rumen using plant secondary metabolites (22) or by feeding dry leaves of eucalyptus (23). However, the associative effects of eucalyptus oils and mulethi (*Glycyrrhiza glabra* L.) root powder in modulating rumen fermentation and animal performance in Murrah buffalo (*Bubalus bubalis*) have not been studied. There remains a need to identify a suitable blend of feed additives that are effective in reducing the ammonia production rate in rumen without affecting feed digestion and animal performance. The present study was conducted to identify and validate a blend of plant-based feed additives that can reduce the rate of ruminal ammonia production without affecting feed digestion in rumen and thus can improve performance and feed efficiency of buffaloes.

MATERIALS AND METHODS

Screening of Blends of Feed Additives

Four blends of feed additives comprising graded doses (5, 10, 15, and 25 μL) of eucalyptus oil and a fixed quantity (15 μL) of aqueous extract of mulethi (*Glycyrrhiza glabra* root; commonly known as licorice or sweet wood) roots were prepared and tested for their suitability to modulate *in vitro* rumen fermentation for reducing ammonia-nitrogen and methane production without impeding feed digestion. The blends were prepared based on a series of preliminary *in vitro* rumen fermentation experiments with graded doses of eucalyptus oil and aqueous extract of mulethi roots individually (24). The eucalyptus oil (80% purity) used for *in vitro* and *in vivo* rumen fermentation studies were purchased from Sigma-Aldrich Ltd. (Bangalore, India). Eucalyptus oil (60% purity) used for the *in vivo* feeding trial on growing buffaloes was purchased from Central Drug House, Delhi, India. Roots of mulethi were purchased from a local market at Hisar, Haryana, India were dried and ground to prepare extract (233.6 g/ L of distilled water) for *in vitro* studies.

Collection of Buffalo Rumen Fluid

Rumen fluid was collected after 2 h of morning feeding from four rumen fistulated Murrah buffaloes (body weight, 700 ± 10.2 kg; age, 60 ± 3 months) fed on a roughage-based total mixed ration (wheat straw: concentrate mixture; 60:40). Four times weekly, they were offered 1 kg (DM basis) of green fodder to meet the requirements for fat-soluble vitamins. The diet was offered twice a day (10:00 and 14:30 h) by dividing into equal amounts, ensuring *ad libitum* feed intake.

In vitro Rumen Fermentation

Substrate (400 mg ± 5 mg oats hay) along with each blend of additive were incubated with 40 mL of buffered rumen inoculum (buffer and strained rumen liquor in a 3:1 ratio) in calibrated glass syringes (Häberle Labortechnik, Lonsee-Ettlenschiess, Germany) of 100 mL capacity and incubated at 39°C for 24 h (25). Three syringes without substrate were incubated as blanks. The

incubation procedure was repeated twice, with three replicates for every treatment.

Estimation of Gas and Methane Production

After 24 h of incubation, the gas production was recorded by the displacement of the syringe piston. The net gas produced due to fermentation of substrate was calculated by subtracting gas produced in the blank syringes from treatments. Methane in the headspace gas was estimated as described earlier (26).

Short-Chain Fatty Acid Estimation

After 24 h of incubation, the supernatant (1 mL) of each syringe content was taken in a microcentrifuge tube containing 0.20 mL of metaphosphoric acid (25%, v/v). The mixture was incubated for 2 h at room temperature and centrifuged at 5,000 g for 10 min. The clear supernatant was then collected and kept at −20°C for subsequent use. For analysis, 1 μL of the supernatant was injected into a gas chromatograph equipped with a flame ionization detector (FID) and glass column packed with chromosorb as described earlier (26).

In vitro Dry Matter Degradability

The content of the syringes was transferred to spoutless beakers (500 mL) by repeated washings with neutral detergent solution. The contents were refluxed for 1 h and the residues were collected in preweighed sintered crucibles (G1). After drying of residues at 60 °C, neutral detergent fiber content was also estimated. True digestibility was calculated as described earlier (27).

Estimation of Ammonia Nitrogen

For estimation of NH_3-N concentration, the Conway disc method was utilized as described in detail earlier (26), where 1.0 mL each of supernatant from syringe contents and a saturated solution of Na_2CO_3 were kept in the opposite direction of outer chamber of the Conway disc and 1.0 mL of 2% boric acid solution with mixed indicators was kept in the inner chamber. The disc were capped after proper greasing and moved slowly by keeping them on the palm of the hand for proper mixing of the solutions of the outer chamber before being placed in an incubator at 39°C for 3 h. After incubation, the boric acid in the inner chamber was titrated with N/100 H_2SO_4 to obtain the NH_3-N concentration. Three blanks were included for correction of NH_3-N produced from the buffered rumen fluid in each run, and two runs were performed for each sample to obtain the representative value.

Evaluation of the Effect of Dosing With the Best Additive Blend on *in vivo* Rumen Fermentation

Four adult rumen fistulated buffaloes were fed a roughage-based diet (concentrate level 40% and wheat straw 60%). Animals were dosed with the best blend (blend-1) of additives for 24 days. In the *in vitro* trial, a satisfactory result was found with blend-1, i.e., a mixture of 5 μL of eucalyptus oil with 15 μL of mulethi root extract (5.84 g of mulethi/25 mL DW) per 40 mL of *in vitro* incubation fluid. This dose was utilized for calculating the dose in fistulated buffaloes of 700 kg BW assuming rumen volume of 10% of BW and 20% higher than the calculated dose to take care of calculation error and the dynamic system. Based on these calculations fistulated buffaloes were fed with a blend of 10.5 mL of eucalyptus oil of 80% purity and 7.35 g of mulethi powder daily during the dosing period. The eucalyptus–mulethi blend (EMB) was mixed with a portion of concentrate mixture and offered at 10:00 h daily. The animals consumed the entire concentrate mixture containing the feed additive within 15–20 min of offering. Sampling of rumen content was done over 3 consecutive days after 21 days post-dosing. Ammonia and short-chain fatty acid (SCFA) levels in rumen fluid were assayed as per the procedure described earlier.

Estimation of Ruminal Enzymes

Rumen liquor was centrifuged at 12,000 *g* at 4°C for 15 min and the supernatant was utilized for estimation of enzymes. Activities of carboxymethyl cellulase (endo-1,4 β-glucanase), xylanase (endo-1,4 β-xylanase), β-glucosidase (β-D-glucoside glucohydrolase), and acetyl esterase (acetic-ester acetylhydrolase) activities were estimated as described earlier (27). Protease activity was measured with azocasein (Sigma Chemical Co., St. Louis, MO, USA) as substrate as described earlier (28). The protein content of the enzyme sample was estimated as per Lowry et al. (29).

Chemical Analysis of Samples

Feed samples were analyzed for dry matter (930.15), ash (942.05), crude protein (988.05), and fat (920.39) by the Association of Official Analytical Chemistry (AOAC) procedures (30) and fiber fractions (neutral detergent fiber [NDF] and acid detergent fibre [ADF]) according to Van Soest et al. (31). NDF was analyzed without decalin, sodium sulfite, and α-amylase and expressed with residual ash.

Extraction of DNA and qPCR-Based Quantification of Different Rumen Microbial Groups

Metagenomic DNA was extracted from the rumen content of each fistulated buffalo (32). The DNA quality was evaluated using agarose gel (1%) electrophoresis, and DNA yield was quantified using a nano-drop spectrophotometer (Invitrogen Corporation, Carlsbad, CA, USA). The DNA samples were stored at −20°C until analysis. The abundances of total archaea and select bacterial species were quantified using SYBR Green-based quantitative real time-PCR (qPCR) using an Applied Biosystem (StepOnePlus) real-time PCR machine (33). To reduce variations, the qPCR assay for each species or group of microbes was performed in triplicate for both the standards and the metagenomic DNA samples using the same master mix and the same PCR plate. The 10-μL master mix contains 5 μL of Power SYBR Green master mix (Invitrogen Singapore Pte. Ltd., Singapore), 1 μL of forward primer, 1 μL of reverse primer, 1 μL of template DNA, and 2 μL of nuclease-free water. The real-time qPCR primers used in this study are listed in **Table 1**. Cycling parameters for qPCR consisted of initial denaturation at 95°C for 10 min for 1 cycle, 40 cycles of amplification (denaturation at 95°C for 15 s, annealing at annealing temperature of the respective primer pairs for 10 s,

TABLE 1 | Primers used for real-time qPCR.

Primer name	Sequence (5′→ 3′)	Expected amplicon size (bp)	Annealing temperature (°C)	References
Total bacteria	F-CCTACGGGAGGCAGCAG R-ATTACCGCGGCTGCTGG	194	60	Singh et al. (34)
Ruminococcus albus	F-CCCTAAAAGCAGTCTTAGTTCG R-CCTCCTTGCGGTTAGAACA	175	55	Singh et al. (34)
Total archaea	F-GGATTAGATACCCSGGTAGT R-GTTGARTCCAATTAAACCGCA	173	56	Hook et al. (35)
Total anaerobic fungi	F-GAGGAAGTAAAAGTCGTAACAAGGTTTC R-CAAATTCACAAAGGGTAGGATGATT	120	56	Lwin et al. (36)
Butyrivibrio fibrisolvens	F-ACCGCATAAGCGCACGGA R-CGGGTCCATCTTGTACCGATAAAT	65	62	Stevenson and Weimer (37)
Megasphaera elsdenii	F-GACCGAAACTGCGATGCTAGA R-CGCCTCAGCGTCAGTTGTC	130	57.8	Bekele et al. (38)

and elongation at 60°C for 50 s with data acquisition at the 60°C step) and melting curve (95°C for 2 min, 60°C for 15 s, and then increasing temperature at the rate of 0.2°C every 5 s up to 95°C and measuring the fluorescence of the melting amplicon at 5-s intervals). Nonspecific primer amplification was assessed by the inclusion of no template controls. qPCR products were checked though agarose gel electrophoresis for the presence of a single band/product. The absolute abundances were expressed as *rrs* gene copies/mL of sample.

Evaluation of the Best Blend of Additives (EMB) on Performance of Growing Buffaloes

Twelve growing buffalo calves (avg. BW 137.5 ± 9.2 kg, 8–12 months old) were selected from an animal farm and assigned randomly to two groups of six each. All the experimental calves were fed a diet containing wheat straw and concentrate mixture (crude protein [CP] 20.04%) in accordance with the nutritional requirement for maintenance and growth (39). One group (control without any additives [CON]) received a control diet (40:60 roughage:concentrate) and the other group (EMB) received the control diet along with feed additive containing eucalyptus oil–mulethi blend (EMB). Each buffalo calf in the EMB group was fed a blend of 2 mL of eucalyptus oil (60% purity) and 1.05 g of mulethi root powder per 100 kg BW by mixing with a small portion of concentrate mixture (**Figure 1**) offered at 10:00 h daily. The animals consumed the concentrate mixture containing additive within 15–20 min of offering. The feeding trial was continued for ∼3 months (84 days) and one digestibility trial of 6 days was performed at the mid-phase of the feeding trial to assess digestibility of nutrients by total collection of feces. Parameters studied were growth rate, feed intake, and digestibility of nutrients. Blood samples were collected from the jugular vein of each experimental calf at 0, 30, 60, and 84 days of feeding trial for estimation of blood biochemical parameters (alanine aminotransferase [ALT], aspartate aminotransferase [AST], total proteion, albumin, globulin, triglyceride, cholesterol, and urea) with commercial biochemical assay kits (Coral Clinical Systems, Verna, Goa, India) on an automated biochemical analyzer (Coralyzer200, Tulip Diagnostics, Goa, India).

Statistical Analysis

All statistical analyses were performed using SPSS 17.0 (40). All the data from *in vitro* experiments were analyzed by one-way ANOVA in a completely randomized design and the differences between the means were compared by Tuckey's test. Data from *in vivo* studies were analyzed by a *t*-test. The log-transformation of the abundance (*rrs* gene copy numbers/30 ng of DNA) of ruminal microorganisms was done before statistical analysis for improved normality. Differences in mean values were considered significant if $p \leq 0.05$, and trends toward significance were examined at $0.05 < p < 0.10$.

RESULTS

Although all four blends examined in the present study were observed to reduce ($p < 0.001$) the ammonia-N and methane production under the *in vitro* fermentation system, only blend-1 containing the lowest dose of eucalyptus oil (5 μL) and 15 μL of aqueous extract of mulethi roots was found to reduce these parameters without impeding true digestibility of dry matter (TDDM) and SCFA production (**Table 2**). Therefore, blend-1 was found to be best among the others.

Effect of the Best Additive Blend (Blend-1) on *in vivo* Rumen Fermentation, Rumen Fibrolytic Enzymes, and Microbial Population Density

There was an ∼50% reduction ($p < 0.01$) in ammonia-N concentration in the rumen fluid of buffaloes supplemented with EMB in samples of 2 h post feeding (**Table 3**). Although individual SCFA production remained comparable ($p > 0.05$), the ratio of acetate to propionate increased ($p < 0.05$) in EMB-supplemented buffaloes compared to the CON. Although the ruminal enzymes, i.e., carboxymethyl cellulase (CMCase), acetyl esterase, and xylanase levels were not affected ($p > 0.05$) by the supplementation of additive (**Table 3**), there were a trend ($p < 0.10$) in increasing CMCase and xylanase activities. However, the protease and β-glucosidase levels were lower ($p < 0.01$) in the EMB group compared to CON.

FIGURE 1 | Preparation and supplementation of eucalyptus oil–mulethi powder blend to buffalo calves. **(A)** Mulethi roots. **(B)** Eucalyptus tree. **(C)** Grounded mulethi roots by Willy grinder. **(D)** Eucalyptus oil. **(E)** Requisite quantities of Mulethi root powder and eucalyptus oil were placed into a small portion of concentrate mixture and mixed uniformly. **(F)** The premix was placed into the manger and mixed with the other portion of concentrate mixture and fed to buffalo calves.

TABLE 2 | Effects of adding various blends of eucalyptus oil and aqueous extracts of mulethi roots to rumen fluid of buffalo on *in vitro* rumen fermentation and methane and total gas production.

Attributes	NH_3-N (mg/dL)	TDDM (%)	Total gas (mL)	Methane (%)	Acetate (mM/dL)	Propionate (mM/dL)	Butyrate (mM/dL)	A:P
CON	51.3^{c}	79.8^{b}	45.0^{d}	18.7^{d}	14.0^{b}	3.6^{b}	1.7	3.9^{b}
Blend-1	43.8^{b}	78.2^{b}	43.0^{c}	17.7^{c}	12.3^{b}	3.1^{b}	1.1	3.9^{b}
Blend-2	43.4^{b}	75.8^{a}	$41.5^{b,c}$	$16.5^{b,c}$	7.6^{a}	1.9^{a}	0.98	3.9^{b}
Blend-3	$42.9^{a,b}$	74.9^{a}	$40.5^{a,b}$	16.3^{b}	7.9^{a}	1.9^{a}	1.1	4.1^{b}
Blend-4	40.6^{a}	74.1^{a}	39.0^{a}	13.5^{a}	7.4^{a}	1.7^{a}	1.0	4.3^{a}
SEM	1.15	0.89	1.06	0.793	1.25	0.65	0.45	0.27
p-value	<0.001	<0.001	0.001	0.001	0.007	0.003	0.23	0.026

Blend-1, eucalyptus oil (5 μL) + mulethi extract (15 μL); Blend-2, eucalyptus oil (10 μL) + mulethi extract (15 μL); Blend-3, eucalyptus oil (15 μL) + mulethi extract (15 μL); Blend-4, eucalyptus oil (25 μL) + mulethi extract (15 μL); CON, control without any additives. A:P, acetate: propionate; NH_3-N, ammonia nitrogen; SEM, Standard error of mean; TDDM, true digestibility of dry matter. Means followed by different superscript letters (a–d) in a column differ significantly ($p < 0.01$).

The real-time PCR data (**Table 3**) indicated that there were no changes ($p > 0.05$) in number of total bacteria, fibrolytic bacteria, or *Megasphaera elsdenii* and total methanogenic archaea in the supplemented group compared with CON. A trend ($p < 0.10$) in reducing anaerobic fungal population was noted.

Effect of Feeding the Additive Blend on Growing Buffaloes

The comparable ($p > 0.05$) intake of wheat straw or concentrate mixture between the CON and supplemented groups (**Table 4**) indicated that the additive mixture did not affect feed intake or palatability of feeds.

TABLE 3 | Effects of best blend of feed additives (blend-1) supplementation to rumen cannulated buffaloes on ruminal pH, short-chain fatty acids, ammonia-N, enzyme activities, and abundances of microbial population.

Attributes	CON	EMB	SEM	*p*
pH	6.4	6.5	0.12	0.776
SHORT-CHAIN FATTY ACID PRODUCTION (mM/dL)				
Acetate	10.2	7.9	1.00	0.183
Propionate	2.9	1.9	0.38	0.114
Butyrate	1.4	0.81	0.23	0.122
Acetate: propionate	3.5^{a}	4.1^{b}	0.12	0.023
NH_3-N (mg/dL)*	22.2^{b}	11.3^{a}	1.59	0.008
ENZYME ACTIVITIES (mIU/mL)				
Carboxymethyl cellulase (endo-1,4 β-glucanase)	19.3	24.6	1.83	0.060
Xylanase (endo-1,4 β-xylanase)	84.9	73.7	4.12	0.074
β-Glucosidase (β-d-Glucoside glucohydrolase)	16.7^{b}	11.7^{a}	0.76	0.0001
Acetyl esterase (acetic-ester acetylhydrolase)	38.2	39.3	1.14	0.489
Protease	18.2^{b}	7.9^{a}	0.020	0.0001
MICROBIAL ABUNDANCE (log16 S RDNA copy numbers/30 ng of DNA)				
Total bacteria	11.85	11.35	0.520	0.536
Ruminococcus albus	6.76	5.92	0.315	0.132
Fibrobacter succinogenes	7.66	6.81	0.900	0.542
Butyrivibrio fibrisolvens	6.32	6.21	0.225	0.379
Megasphaera elsdenii	4.48	4.83	0.345	0.142
Anaerobic fungi	8.03	7.10	0.256	0.062
Total archaea	7.41	6.34	0.402	0.120

*CON, control group; EMB, eucalyptus–mulethi blend (control diet with a blend of 10.5 mL of eucalyptus oil and 7.35 g of mulethi root powder/h/day); SEM, standard error of mean. *Measured at 2 h post-feeding. Means followed by different superscript letters (a, b) in a row differ significantly ($p < 0.05$).*

TABLE 4 | Growth rate and feed efficiency of buffalo calves supplemented with feed additive.

Attributes	CON	EMB	SEM	*p*
BODY WEIGHT GAIN				
Total gain* (kg)	60.8^{a}	69.4^{b}	3.06	0.049
ADG (g)	724.0^{a}	826.0^{b}	37.5	0.049
DRY MATTER INTAKE (kg/day)				
Concentrate	3.13	3.19	0.19	0.444
Wheat straw	1.45	1.49	0.11	0.25
Total	4.59	4.69	0.29	0.304
FCR	6.37	5.73	0.38	0.057
CP intake (g/day)/g ADG	1.40	1.10	0.11	0.084
TDN intake (g/day)/g ADG	5.14	4.11	0.45	0.115

*CON (control group), diet containing a concentrate mixture and wheat straw EMB; (treatment group) = control diet supplemented with a blend of 2 mL of eucalyptus oil (60% purity) and 1.05 g of mulethi root powder per 100 kg BW per day. ADG, average daily gain; CP, crude protein; FCR, feed conversion ratio (kg dry matter intake/kg gain); SEM, standard error of mean; TDN, total digestible nutrients. *After 84 days. ^{a,b}Mean values bearing different superscripts within a row vary significantly ($p < 0.05$).*

The total gain (kg) in body weight at the end of the feeding trial and average daily gain (ADG, g) was higher ($p < 0.05$) in buffalo calves in the EMB group compared to CON (**Table 4**).

TABLE 5 | Nutrient digestibility and nutritive value of diets fed to buffalo calves supplemented with feed additives.

Attributes	CON	EMB	SEM	*p*
NUTRIENT DIGESTIBILITY (%)				
Dry matter	58.9	59.7	1.40	0.701
Organic matter	59.3	60.3	1.54	0.920
Crude protein	72.2	73.8	1.26	0.378
Ether extract	71.5	75.0	2.08	0.261
Neutral detergent fiber	56.8	58.9	1.55	0.353
Acid detergent fiber	52.3	53.7	1.51	0.503
NUTRIENT DENSITY (%)				
CP	15.7	15.6	0.39	0.793
DCP	11.4	11.4	0.43	0.843
TDN	57.2	58.0	1.24	0.639

CON, control group; CP, crude protein; DCP, digestible crude protein; EMB, eucalyptus–Mulethi blend supplemented group; SEM, standard error of mean. TDN, total digestible nutrients.

The feed conversion ratio (FCR) and efficiency of conversion of dietary protein for body weight gain tended ($p < 0.10$) to be better in the supplemented group compared to CON.

The digestibility coefficient of various nutrients (**Table 5**) did not differ ($p > 0.05$) in animals in both the CON and supplemented groups, indicating that the feed additive did not exert any adverse effect on whole gastrointestinal tract digestibility of nutrients in calves (**Table 5**). The blood biochemical parameters (**Table 6**) in animals of both groups remained comparable ($p > 0.05$) throughout the experimental feeding, although blood urea level tended ($p < 0.10$) to be reduced at the 84th day in EMB-supplemented buffaloes compared to CON.

DISCUSSION

Feed additives of plant origin are gaining importance in the diet of ruminants for their antimicrobial properties to modulate rumen fermentation to increase animal production and reduce environmental pollution. The activity of essential oils including eucalyptus oil depends on the presence of the kinds of functional groups and their proportions. The major secondary metabolite present in eucalyptus oil is 1,8-cineole, which is responsible for the antimicrobial property (41). The inhibition of ruminal hyper-ammonia-producing bacteria (HAB) responsible for deamination of amino acids to ammonia in the protein degradation pathway by eucalyptus essential oils (EOs) could be the reason for lowered ammonia production (42). The reduction of ammonia-N and methane production on *in vitro* incubation of oats hay with eucalyptus oil was also reported by Singh et al. (26). However, in *in vivo* studies involving other EOs, many researchers (43–45) demonstrated similar concentration of NH_3-N in rumen fluid of both control and EO-supplemented animals. A lower ($p < 0.05$) level of NH_3-N was reported by Patra (46) in garlic-fed fistulated buffaloes. While studying the effects of some EOs and their

TABLE 6 | Effects of supplementation of the feed additives on blood biochemical profile of buffalo calves.

Parameters	Days	CON	EMB	SEM	*p*
Urea (mg/dL)	0 day	18.00	15.60	3.21	0.610
	30 day	30.06	27.38	7.42	0.804
	60 day	33.52	32.82	3.87	0.901
	84 day	43.22	30.22	4.51	0.087
Cholesterol (mg/dL)	0 day	70.20	72.88	3.68	0.620
	30 day	82.85	81.55	4.14	0.828
	60 day	86.84	85.9	2.09	0.758
	84 day	82.58	78.22	2.9	0.311
Triglyceride (mg/dL)	0 day	66.78	65.06	4.12	0.775
	30 day	49.38	45.24	3.44	0.419
	60 day	68.2	71.35	4.58	0.338
	84 day	53.3	59.06	5.37	0.470
Total protein (g/dL)	0 day	5.88	5.8	0.206	0.781
	30 day	5.58	5.45	0.116	0.434
	60 day	5.68	5.56	0.181	0.658
	84 day	5.83	5.61	0.183	0.421
Albumin (g/dL)	0 day	0.98	0.98	0.025	1.00
	30 day	0.91	0.85	0.131	0.282
	60 day	0.98	0.98	0.025	1.00
	84 day	1.05	1.03	0.034	0.734
Globulin (g/dL)	0 day	4.9	4.82	0.179	0.749
	30 day	4.66	4.6	0.112	0.683
	60 day	4.7	4.58	0.169	0.637
	84 day	4.78	4.58	0.176	0.440
AST (units/L)	0 day	171.57	178.75	10.16	0.641
	30 day	132.85	131.45	13.01	0.941
	60 day	117.08	125.08	4.51	0.297
	84 day	123.00	118.85	3.99	0.479
ALT (units/L)	0 day	79.92	80.48	4.58	0.933
	30 day	57.52	50.56	2.47	0.081
	60 day	66.12	65.7	2.59	0.912
	84 day	65.46	62.38	2.2	0.353

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CON, control group; EMB, eucalyptus–Mulethi blend supplemented group; SEM, standard error of mean.

main active compounds on rumen fermentation, Busquet et al. (47) demonstrated marked inhibition of NH_3-N concentration at high doses with marginal to no effects at moderate and low doses, respectively. Earlier, Sallam et al. (23) reported a reduction in ruminal ammonia nitrogen concentration on feeding fresh and dry leaves of eucalyptus as compared to alfalfa hay.

Mulethi is known to contain tannins and saponins besides other plant bioactive compounds (48). A substantial reduction of ruminal proteolytic activity as well as ammonia nitrogen concentration in sheep fed condensed tannin (CT) from *Lotus corniculatus* was demonstrated by Min et al. (49). Similarly, a reduced ruminal ammonia nitrogen concentration by supplementation of *Yucca schidigera* (rich in saponin) extract was described in a continuous incubation system (50). As proposed previously (42, 51) for other EOs, the reduction in ammonia nitrogen production by the additive blends used in the present study could be attributed to direct inhibition of HAB, which are low in number but produce ammonia at a much faster rate than other rumen microbes. The current observation that high doses of eucalyptus oils in blends-2,−3, and−4 tested tend to reduce *in vitro* dry matter digestibility, total gas and methane production, and ruminal SCFA production at higher doses but the lower dose (blend-1) may not affect SCFA production is consistent with those of Castillejos et al. (52) and Patra and Yu (53).

The lower ($p < 0.01$) protease activity observed in this study is expected to reduce amino acid degradation in rumen, which in turn will increase the supply of proteins for animals. McInotsh et al. (10) also observed inhibition of some HAB growth with addition of a mixture of EOs. As there was a severe decrease in ammonia concentration in rumen fluid on feeding the additive, it is possible that the additive might have selectively inhibited some of the HAB, including some of the uncultured group, without affecting total bacterial count. In the present study, the methanogenic archaea population tended to decline in the treatment group, which indicated that the identified additive can have an added advantage by reducing methane emission in addition to lower ammonia emission. The increasing ($p < 0.10$) activities of CMCase and xylanase might have contributed toward digestion of feed in the animals, resulting in greater acetate production compared to propionate, thus increasing the acetate to propionate ratio. The quantity and species of HAB varies with the diet. Again the effects of EOs in the stationary phase under *in vitro* conditions and dynamic system of rumen may be different. This may explain the higher reduction of ammonia-N in *in vivo* experiments (10). The trend in reducing rumen anaerobic fungi in EMB-supplemented buffaloes is due to the direct inhibitory effect of EOs on these microbes (54).

In the current study, no adverse effect of the additive on feed intake was observed. In contrast, reduction in feed intake on feeding some of the EOs was reported in other studies that might be related to palatability problems (55). Scant information on the effect of feeding blend of eucalyptus oil plus mulethi on whole gastrointestinal tract digestibility of nutrients is available for comparison. Benchaar et al. (56) and Santos et al. (57) reported similar nutrient digestibility in dairy cows fed (2 g/day) with a mixture of EOs, whereas, higher (13%) dry matter (DM) digestibility was described by Yang et al. (45) in dairy cows supplemented (2 g/day) with juniper berry EOs.

Data on the effect of a feeding blend of eucalyptus oil and mulethi on growth rate are not available for comparison. Although feed intake and nutrient digestibility remained comparable for both the groups, better utilization of nitrogen perhaps increased the ADG in EMB-supplemented calves, which was also evidenced by better FCR ($p = 0.057$) and protein utilization efficiency (1.4 vs. 1.1 g CPI/g ADG; 21.4% increase). In an experiment on beef cattle supplemented (2 or 4 g/day) with a mixture of EOs, Benchaar et al. (56) demonstrated comparable ADG in both control and supplemented groups. However, higher ADG was reported

by Chaves et al. (58) on supplementation (0.2 g/kg of DM) of either cinnamaldehyde or juniper berry EOs in growing lambs. The present results corroborate the description of Khateri et al. (59), in which feeding of a mixture of EOs in ruminants had no effect on blood biochemical parameters; however, a trend to reduce blood urea concentration at a later stage of the present feeding experiment in EMB-supplemented buffaloes could also explain the reduced degradation of protein in rumen.

CONCLUSIONS

The blend of a low dose of eucalyptus oil plus mulethi root extract was found to have a potential to reduce the rate of ruminal ammonia production without affecting feed digestion and thus improving feed efficiency. A dose of 10.5 mL of eucalyptus oil of 80% purity and 7.35 g of mulethi root powder daily can reduce ruminal ammonia production by 50% in adult buffaloes weighing 700 kg without affecting feed digestibility. *In vivo* studies in growing buffaloes confirmed that this feed additive can improve growth performance and tend to improve protein efficiency and feed utilization efficiency in buffaloes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Experimental protocol was approved by the ICAR-CIRB animal ethics committee (IEAC protocol no. 1/2016).

AUTHOR CONTRIBUTIONS

YC and SP: conceived and designed the experiment. YC, SP, and AD: collected raw materials and performed the experiment. AD and SD: analyzed the data and interpreted the results. SP and AD: wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The work was supported by ICAR-Central Institute for Research on Buffaloes, India.

ACKNOWLEDGMENTS

We also thankfully acknowledge the assistance of Dr. Andonissamy Jerome in analysis of blood biochemical parameters.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00464/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 02 September 2020
doi: 10.3389/fvets.2020.00518

Inferring Relationship of Blood Metabolic Changes and Average Daily Gain With Feed Conversion Efficiency in Murrah Heifers: Machine Learning Approach

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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: 26 March 2020
Accepted: 06 July 2020
Published: 02 September 2020

Citation:
Sikka P, Nath A, Paul SS, Andonissamy J, Mishra DC, Rao AR, Balhara AK, Chaturvedi KK, Yadav KK and Balhara S (2020) Inferring Relationship of Blood Metabolic Changes and Average Daily Gain With Feed Conversion Efficiency in Murrah Heifers: Machine Learning Approach. Front. Vet. Sci. 7:518. doi: 10.3389/fvets.2020.00518

Machine learning algorithms were employed for predicting the feed conversion efficiency (FCE), using the blood parameters and average daily gain (ADG) as predictor variables in buffalo heifers. It was observed that isotonic regression outperformed other machine learning algorithms used in study. Further, we also achieved the best performance evaluation metrics model with additive regression as the meta learner and isotonic regression as the base learner on 10-fold cross-validation and leaving-one-out cross-validation tests. Further, we created three separate partial least square regression (PLSR) models using all 14 parameters of blood and ADG as independent (explanatory) variables and FCE as the dependent variable, to understand the interactions of blood parameters, ADG with FCE each by inclusion of all FCE values (i), only higher FCE values (negative RFI) (ii), and inclusion of only lower FCE (positive RFI) values (iii). The PLSR model including only the higher FCE values was concluded the best, based on performance evaluation metrics as compared to PLSR models developed by inclusion of the lower FCE values and all types of FCE values. IGF1 and its interactions with the other blood parameters were found highly influential for higher FCE measures. The strength of the estimated interaction effects of the blood parameter in relation to FCE may facilitate understanding of intricate dynamics of blood parameters for growth.

Keywords: buffalo, blood, feed conversion efficiency, partial least square regression, prediction models

INTRODUCTION

Feed efficiency is a multifactorial functional trait reflecting the energy balance of a particular animal, which determines its overall productivity. Feed cost constitutes 70% of total input of production system profits; thus, improvement in feed utilization capacity of an animal would be very profitable (1). Expensive feed costs for milk/beef producers can be minimized by increasing feed efficiency. Beef cattle, utilizing feed efficiently, showed substantially curtailed feed

consumption for comparable productive performance in contemporary animals (2). Residual feed intake (RFI) has been an accepted measure of feed utilization efficiency of animals, which defines the differences in actual and expected feed intake due to the different background energy requirements of different animals (3). Feed-efficient animal displays lower RFI, an attribute inscribed with moderate heritability of 0.15 and repeatability as 0.53 (4) and is also being used in selection programs (5). It is having limited use in industry due to its time-consuming monitoring and heavy capital investment, consequently emphasizing on the need to explore alternate approach to infer feed efficiency, as blood parameters.

The knowledge of different sources of variation that cause physiological differences among animals in terms of feed efficiency, mainly residual feed intake (RFI), is still incomplete. Variations in blood parameters and metabolic characteristics reflect appreciably a part of total feed efficiency variation in animals (6, 7). A thorough study of all possible processes related to this variation, if it does not lead to an efficient early selection, at least would be useful for deducing genotypes selectively for RFI/FCE. Blood metabolic markers associated with feed conversion efficiency were earlier used to enhance profitability (7, 8) of yearling beef bulls (9) and crossbred heifers (10), wherein the level of FCE was extrapolated on the scale of energy substrates as blood metabolite(s), i.e., glucose, triglycerides, urea, creatine phosphokinase as protein metabolite, total plasma protein, and aspartate aminotransferase, which in turn are influenced by the hypothalamus–pituitary–adrenal axis (11, 12). Several types of potential proxies for RFI, using energy metabolism (13), hepatic mitochondrial function (14), and visceral organ metabolism (15), have been identified to monitor feed efficiency in other species. Change in urea level has been associated with RFI (16), which is attributed to the rate of degradation or synthesis of protein (17), reflecting liver function and metabolic activity of the digestive tract while generating almost 40 to 50% of the total energy channeling 1.45% in animal body weight (18, 19).

An insight into the relationship of various blood parameters with ADG with FCE can shed light on physiological dynamics underlying the metabolic changes, using machine learning approaches (20–22). The biological closeness between feed efficiency and the animal's ability to convert feed nitrogen (**N**) into animal protein, i.e., N-use efficiency or N partitioning and protein turnover across individuals (23), has been used for predicting RFI in growing cattle due to the difference in rates of amino acid transamination (24).

Studies in Indian *Bubalus bubalis* to this effect are scanty. Exploring indirect markers as discriminatory change in blood attributes would be useful to frame predictive models to establish genetic markers for optimization of multifunctional complex traits as FCE. It will help in selecting efficient buffalo aptly christened "Black Gold" (25, 26), which contribute more than 22% toward worldwide demand of milk, meat, and hide (27). The mathematical model enabling the user to explore the relationship between nutrition (glucose, insulin, and IGF1 system) and reproduction is recently developed (28) for cattle as an early attempt toward developing *in silico* feeding strategies, which may reduce animal experiments eventually.

The present study is an attempt to deduce the intricate relationship between the changing dynamics of circulatory metabolites and the level of feed, utilizing efficiency in order to find out proxy indicators other than RFI and developing best-fit models to predict feed efficiency by machine learning. Blood parameters and ADG remained as predictor variables and FCE as the response variable to obtain models. Least square means were used to develop partial least square regression (PLSR) models for FCE predictions.

MATERIALS AND METHODS

Ethical Statement

All animal experiments were performed under permission and review of the Institutional Animal Ethics Committee (IAEC) (Reg. No. 406/GO/RBI/L/01/CPCSEA). Experimental heifers ($n = 42$) used in the present study were selected at ICAR-Central Institute for Research on Buffaloes Hisar, Haryana, Govt. of India, buffalo farm to determine the levels of variation in RFI over animals under study of more than 100 days. RFI was determined as the difference in actual and predicted dry matter intake (DMI).

Animals and Samples

Forty-two growing Murrah female buffalo calves (initial weight, 155 ± 4.6 kg; initial age, between 9 to 11 months) were utilized for the study. The buffaloes were vaccinated and treated to eliminate external and internal parasites before initiation of the study. The buffaloes were fed individually on diets comprising *ad lib* (allowing residues approximately at 10% of total daily dry matter intake) green Jowar (*Sorghum vulgare*) fodder and a fixed quantity of concentrate mixtures (50% of expected dry matter intake of individual animal). The diet was formulated to meet nutrient requirements as per buffalo feeding standards developed by Paul and Lal (29).

The quantities of offered fodder and concentrate were adjusted at fortnightly intervals depending on dry matter intake of the preceding fortnight. The residual fodder and feed were removed, weighed, and sampled for dry matter (DM) estimation before offering the next day's concentrate allowance. The DM of offered and residue fodder samples was estimated on a daily basis. The offered and residue samples of feeds and fodders were pooled at monthly intervals for chemical analysis (**Table 1**).

TABLE 1 | Chemical composition of feeds[a] (g/kg).

	Concentrate mixture[b]	Jowar fodder
Organic matter	865	943
Crude protein	241.5	60.7
Ether extract	42.9	29.9
Crude fibee	94.5	322

[a] *Values represent hexaplicate assays of each material.*
[b] *Ingredient composition of concentrate mixture: maize grain, 175 g/kg; barley grain, 175 g/kg; wheat bran, 270 g/kg; mustard cake, 200 g/kg; cotton seed cake, 150 g/kg; mineral mixture, 20 g/kg; common salt, 10 g/kg.*

Daily concentrate and fodder DM intake were recorded for each calf. Body weight changes were recorded every 2 weeks. The feeding trial continued for 96 days. One digestibility trial (comprising a 6-day collection period) involving four animals was conducted after about 65 d of feeding trial to ascertain the nutritive value of diet. The offered feeds and residues of the previous day were recorded; samples of both were collected daily and pooled throughout the experimental periods per animal for analysis. The feces voided was immediately collected and placed in covered bins animal-wise. The amounts of feces voided daily were weighed and thoroughly mixed in a pail, and an aliquot (1 g per kg fresh feces) was mixed with 15 ml of 20% H_2SO_4 and kept for N estimation. Another portion of the aliquot (30 g per kg (minimum of 100 g) fresh feces) was kept for drying at 70°C in a hot air oven for the estimation of dry matter and other proximate composition. Representative samples of feed offered, residues left, and feces voided were analyzed to determine nutrient digestibility. Feed and fecal samples were analyzed for dry matter (proc. # 930.15), ash (proc. # 942.05), crude protein (proc. # 988.05), and fat (proc. # 920.39) by procedures of AOAC (1990).

Methodology for Measuring Residual Feed Intake (RFI) in Buffalo Calves

The BWs of individual animals, recorded at the time of initiation and completion of the trial, were compared to determine the average daily weight gain (ADG):

$$\text{Average Daily Gain} = \text{Total weight gain during trial} \div \text{No. of days}$$

Daily feed intake was recorded for each animal, and body weight was taken fortnightly. The average DMI for the 112-day feeding period was regressed on average metabolic body weight (BW 0.75) and average daily gain (ADG) (30). RFI was computed for each animal and was assumed to represent the residuals from a multiple-regression model regressing dry matter intake (DMI) on ADG and average metabolic BW (MBW) (BW 0.75). The actual DMI minus the predicted DMI corresponds to the RFI. The base model used was $Yj = \beta 0 + \beta 1MBWj + \beta 2ADGj + ej$, where Yj is the DMI of the jth animal, β0 is the regression intercept, β1 is the regression coefficient on MBW, β2 is the regression coefficient on ADG, and ej is the uncontrolled error of the jth animal (RFI). A more efficient animal has a negative RFI (observed feed intake is less than predicted feed intake), and a less efficient animal has a positive RFI (observed feed intake is greater than predicted feed intake). The allocation of animals over the two subgroups of low and high conversant was based on estimated RFI. High and low feed conversant animals were identified based on residual feed intake (RFI) as a measure FCE. The relation between blood analytes and feed efficiency in terms of RFI assigned to individual heifer was established. The study hypothesized low dry matter intake, translated into low residual energy intake, as indices of high energy conversant and so the productivity of an animal.

Sampling

Blood samples (10 mL) were collected at each instance of initiating the trial (day 0), followed by days 30, 60, and 90 of the 96-day feeding trial at h 9.00 from 42 growing heifers in the study during July to October from the jugular vein in a serum clot-activated vacutainer (VACUETTE®). After collection, samples were centrifuged at 3,000 rpm, 4°C for 15 min. Serum was separated and stored at −20° C until analyzed. Blood serum estimates of urea, total protein, albumin, cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides, lactate dehydrogenase, serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), and phosphorus were computed using an automated biochemical analyzer (Coralyzer 200, Tulips Diagnostics, India) and commercial kits (Coral Clinical Systems, India). Serum insulin-like growth factor-1 (IGF1), triiodothyronine (T_3), & thyroxine (T_4) levels were estimated using ELISA kits (Sincere Biotech Co., Ltd. Beijing). The intra-assay and inter-assay coefficients of variation were ≤9% and ≤15%, respectively.

Blood Parameter Dataset

Blood parameters were measured in all the samples collected from 42 heifers at four different intervals, i.e., at start of the trial (day 0), followed by three more collections on the 30th, 60th, and 90th days of the feeding trial. Day-wise outlier detection for every blood parameter was applied using the box and whisker plot method in R statistical language (31), picking 32 observations out of 42. In box and whisker plots, the central mark is the median (q2); the edges of the box were the 25th q_1 and 75th q_3 percentiles. Points were drawn as outliers if they were larger than q_3+W (q_3-q_1) or smaller than q_1-W (q_3-q_1), where $W = 1.5$ (three states of vectors q1, q2, q3). Means over 4 intervals of each of the blood parameters were employed including the values of outliers imputed by the Markov chain Monte Carlo (MCMC) method (32) for 32 out of 42 animals in trial, with their corresponding age and ADG to compute the potential of feed utilization function in altering intermediary metabolic differences of high and low feed conversant based on the RFI (−0.437 to 0.359) determined in this study. The difference in average DM intake between the heifers of two energy-utilizing subgroups was recorded as 100 g per day. The av. body weight (BW) gain over 42 heifers was 45 kg during the feed trial with an average initial BW of 155 kg ranging between 96 and 214, attaining final BW as 200 kg (ranging between 147 and 254 kg). The average daily weight gain (ADG) remained 590 g/day, ranging between 382 and 807 g/day. Ten animals were selected in each of the high and low feed conversant subgroups, based on daily DMI (between 3.3 and 6.0 kg) to analyze the variation in blood attributes over two feed utilizing levels in heifers.

Machine Learning Platform

All the machine learning algorithms were implemented using the Java-based Waikato Environment for Knowledge Analysis (WEKA) data mining software package (33) available as an open source. Machine learning models can account for complexity

of predictor and response variable relationship over correlation analysis, infested with the limitation of determining only linear relationship positive, negative, or none between variables, without showing causation. An individual heifer with a set of 14 blood parameters and estimated value of RFI (FCE) was described as the response variable.

SMO reg uses support vector machines for regression (34). **IBK** assigns an outcome value as the nearest neighbor-based algorithm, by taking the average of the numerical target of the K nearest neighbor (35). **Locally Weighted Learning (LWL)** algorithm uses an instance-based algorithm to assign instance weights, which are then used by a base classifier for prediction (35). **Random Forest (RF)** is an ensemble learning algorithm consisting of a number of individual decision trees. At each node of the decision tree, a bootstrapped sample of training instances is evaluated along with a random subset of features followed by combination of decision outcome of individual decision trees 23 (36).

Isotonic Regression is a repressor for a dataset of low-level oscillations (noise), enabling capture of the internal dynamics contrary to obtaining false high scores by considering the slope as a straight line in linear regression. It minimizes the function

$$f(x) = \sum_{i=1}^{n} W_i \left(Y_i - \hat{Y}_i\right)^2$$

where $Y_i = y_1, y_2, \ldots\ldots, y_n$ are observed responses and $_{1,2,}\ldots\ldots,$ $_n$ are the unknown response values and W_i are the positive weights, fit for the least square method for monotonically increasing/decreasing functions (35).

Additive Regression has been used to generate accurate regression (37, 38) at each iteration, the residuals left over as a meta-classifier in the preceding iteration, to fit the model.

Partial Least Square Regression (PLSR) (39, 40) is a multivariate statistical procedure to build explanatory and predictive models to analyze multiple-response (dependent) and multiple explanatory (independent) variables, where high multicollinearity in small sample size ceases reliable conclusions due to classical regression solution. The algorithm was applied using XL stat (trial version).

Performance Evaluation Parameters

Performance of the machine learning algorithms was evaluated using 10-fold cross-validation and leave-one-out cross-validation methods. The dataset was divided into ten equal divisions in 10-fold cross-validation, where 9 divisions are used for training and the one left division is used for testing. This process is repeated till each fold is used once for testing. Leave-one-out cross-validation (LOOCV) is a special case of K-fold cross-validation, where each sample is used once for testing. LOOCV is considered to be the most objective test and is preferred for small data-set instances (41–46). The performances of the machine learning algorithms are further evaluated using performance evaluation metrics—correlation coefficient, mean absolute error, and root mean square error.

Mean Absolute Error (MAE)

The mean absolute error (MAE) is defined as the difference between values predicted by a model and the values actually observed from the real environment. It is derived from the unaltered magnitude (absolute value) of each difference

$$\text{MAE} = \frac{\sum_{i=1}^{n} |X_{obs,i} - X_{model,i}|}{n}$$

Where, n = the number of samples

$X_{obs,i}$ = observed value of FCE

$X_{model,i}$ = predicted value of FCE

Root Mean Square Error (RMSE) is also known as the root mean square deviation, calculated as the difference between the values predicted by a model and the values actually observed from the real environment (FCE) that is being modeled.

$$\text{RMSE} = \sqrt{\frac{\sum_{i=1}^{n} (X_{obs,i} - X_{model,i})^2}{n}}$$

$X_{obs,i}$ = observed value of FCE

$X_{model,i}$ = predicted value of FCE

Model Quality Indices for PLSR

The quality of the PLSR model was evaluated by the three model quality indices, i.e., Q^2 cumulated (Q^2 cum), R^2Y cumulated (R^2Y cum), and R^2X cumulated (R^2X cum). Q^2 cumulative gives global goodness of fit and the predictive accuracy of the first components.

$$Q^2cum(h) = 1 - \prod_{j=1}^{h} \frac{\sum_{k=1}^{N} PRESS_{kj}}{\sum_{k=1}^{N} SSE_{k(j-1)}}$$

The index involves the calculation of PRESS statistic (using cross-validation) and the sum of squares of errors (SSE) with one less component. R^2Y cum gives the correlation between the explanatory (independent) variables with the components and R^2X cum correspond to the correlations between the dependent variables with the components.

RESULTS AND DISCUSSION

The objective of the present study was to have an insight into physiological dynamics involving "pattern change" in various blood parameters in respect of average daily weight gain (ADG) and feed conversion efficiency (FCE) depicted as residual feed intake (RFI). The latter was estimated as the difference of actual and predicted DM intake (DMI) for each individual animal. Variation over mean BW, ADG, DMI, and residual feed intake was determined in heifers.

Blood Attributes

Repeated-measure ANOVA estimates of all the blood parameters covered under the study are depicted in **Table 2**. Levels of total protein, triglycerides, SGOT, and phosphorus in blood serum

TABLE 2 | Descriptive statistics of all blood parameters, ADG along with FCE ($n = 32$).

Blood parameters	Minimum	Maximum	Mean	Std. deviation	Individual variation (*F*-value, $P \leq 0.05$)
Urea (mg/dl)	14.400	29.250	22.861	2.653	0.31, 1.000
Total Protein (g/dl)	4.725	6.700	5.705	0.506	0.89, 0.638
Albumin (g/dl)	1.575	2.550	2.026	0.208	0.73, 0.837
Cholesterol (mg/dl)	53.000	80.725	63.706	7.026	0.85, 0.684
LDL (mg/dl)	17.575	43.825	30.643	5.657	0.99, 0.488
HDL (mg/dl)	42.350	61.675	51.931	4.723	0.35, 0.999
TG (mg/dl)	31.900	46.200	38.605	3.110	0.78, 0.780
LDH (U/l)	676.400	1221.725	827.352	105.570	0.20, 1.000
SGOT (U/l)	52.250	87.850	66.127	8.183	0.22, 1.000
SGPT (U/l)	35.175	50.100	42.125	3.504	0.15, 1.000
Phosphorus (mg/dl)	5.100	6.700	5.875	0.408	0.62, 0.934
IGF-1 (ng/ml)	96.535	183.234	127.335	20.232	0.40, 0.997
T-3 (ng/ml)	1.432	1.952	1.639	0.128	0.46, 0.990
T-4 (ng/ml)	44.597	160.357	103.794	26.210	1.69, 0.029*
ADG (kg)	0.369	0.729	0.549	0.097	
FCE (RFI)	−0.204	0.359	0.008	0.133	

*Significant at $p < 0.05$.

are comparable with earlier reports (47). The level of albumin and cholesterol in serum of heifers was estimated to be lower, but LDH and SGPT levels were higher than corresponding values reported in adult buffaloes. Higher energy status of heifers than adult buffaloes corroborates with higher energy status during active growth. Significant individual variation was recorded in respect of blood thyroxin, a growth regulator in these animals.

Variation in Levels of Blood Attributes and Their Test of Significance Over Feed Utilizing Efficiency

The two-sample *t*-test was carried out on estimated mean values of each of the blood attributes to record the variation in circulatory levels in respect of the difference in two subgroups, each having ten animals bearing extremely high or low feed conversion efficiency, i.e., residual feed intake (RFI). Equality of the estimated means was derived from samples collected on the initial day (day 0) followed by the 30th, 60th, and 90th days of the feeding trial, for every blood attribute tested in the study by the two-sample *t*-test, comparing two categories of high and low feed utilization efficiency animals (**Table 3**). Blood urea and SGPT levels differed in animals of high and low feed conversion efficiency subgroups initially at the time of initiating the trial, which was recorded non-significant later during the trial, indicating the uniform dietary status of study animals under institute management during the trial. However, total serum protein differed between animals of two feed efficiencies significantly on day 1 (<0.05) and day 90 (<0.001) of the trial, indicative of different pathways of protein utilization for the same productivity in two subgroups of heifers. While comparing the serum level of blood attributes between low and high feed conversant heifers, significant elevation was recorded, respectively, in albumin 1.8/2.1 ($p < 0.05$); cholesterol, 47.9/60.8 ($p < 0.001$); LDH, 534/721 ($p < 0.05$); SGOT, 35.7/46.8 ($p < 0.001$); T_3, 1.5/1.8 ($p < 0.05$) on day 90; and T4 ($p < 0.001$) on day 30 of the feeding trial.

TABLE 3 | Test of significance (computed t stat values) of blood metabolite(s) over different feed utilizing heifers ($n = 20$).

Period during feed trial	Zero d	30 d	60 d	90 d
Urea (mg/dl)	0.315	**2.078***	1.573	1.547
Total protein (g/dl)	**2.171***	0.886	0.619	**4.596****
Albumin (g/dl)	0.698	0.211	1.582	**2.028***
Cholesterol (mg/dl)	0.265	0.455	**1.938***	**3.182****
LDL (mg/dl)	0.650	0.245	1.191	0.196
HDL (mg/dl)	0.279	1.358	1.136	**4.444 ****
TG (mg/dl)	0.709	0.305	**3.152****	0.775
LDH (U/l)	0.341	0.131	**2.232***	**1.833***
SGOT (U/l)	1.344	0.985	**2.098***	**3.123****
SGPT (U/l)	**2.995****	0.925	1.4631	1.506
Phosphorus (mg/dl)	**1.702***	0.810	1.857	1.072
IGF-1 (ng/ml)	0.734	0.146	**2.449****	0.111
T-3 (ng/ml)	1.096	**2.203***	0.300	−0.377
T-4 (ng/ml)	0.607	**2.454****	0.775	0.775

*Significant at ($p > 0.05$).
**Significant at ($p > 0.001$).
Bold values indicates the significant.

Total protein was found significantly higher (<0.05) in animals of the higher-efficiency subgroup. Insulin is known to diverge from IGF-I along with growth hormone (GH), where the function of these hormones is known to link the regulation of both nutrient availability and its repletion, continuing to provide adequate signals and substrate for growth (48). Pro-insulin

and IGF1 modulate carbohydrate metabolism, which stimulates glucose transport and inhibits insulin sensitivity. Low IGF I estimated in efficient conversant is found to be associated with metabolic deviations related to lower cholesterol at day 60 ($p < 0.05$) and day 90 ($p < 0.001$) and lower triglycerides on day 60 ($p < 0.001$) during the feed trial in the present study (**Table 2**) instead of hyperlipidemia and hyperinsulinemia reported in other studies (49). A higher level of IGF1 in the subgroup of less efficient energy-utilizing animals indicates a higher stimulus to body for making metabolic changes for growth; however, secretion of higher IGF1 in circulation might also suggest inhibitory feedback influence on the GH/pituitary axis, thus affecting feed utilization efficiency. Significantly low ($p < 0.001$) SGPT (59.5 ± 0.64 U/L) was recorded in less efficient animals compared to efficient animals having a higher level of 67.72 ± 0.78 U/L, which corroborates with other reports in cattle (7, 24), further indicating gluconeogenesis as the preferred energy pathway in efficient animals. A significant ($p < 0.05$) difference in serum urea level of less efficient vs. highly efficient animals, i.e., 23.83 ± 0.35 vs. 20.94 ± 0.51 mg/dl (on day 30 of trial), indicates the effect of change in season during this particular period of July to August months covered during the trial in the present study. Onset of rains in the month of August may influence the dietary patterns in animals along with climate change. Also, downregulation of different transaminases with corresponding lowering in serum urea levels was reported by other researchers (24) in cattle. Contrary to earlier studies (7) performed in beef cattle, serum SGOT levels were recorded to be higher in efficient buffalo heifers than in the inefficient subgroup of animals in the present study. The difference between the species in respect of SGOT levels in two feed efficiency subgroups of buffalo heifers may be attributed to the difference in rumen microbiota and functioning of liver of both species (50). Efficient heifer calves also tended to have a lower concentration of T_3 during the performance evaluation ($p < 0.05$), compared to the efficient heifer calves as reported earlier (23). It is also documented that during growth, T3 has a synergistic relationship with the growth hormone in heifers (51), supporting the argument of metabolic rate differences between heifer calves of distinct feed efficiency classifications.

Relationship of Blood Parameters and Average Daily Gain With Feed Conversion Efficiency

The study of the interaction of the blood parameter in relation to FCE may facilitate understanding of intricate dynamics of intermediary metabolism during growth. A vast variation in physiological levels of blood attributes was observed in heifers. The correlation matrix (**Table 4**) depicts a linear relationship of blood attributes and average daily weight gain [ADG] with feed conversion efficiency [FCE]. Total protein and albumin were observed to have a significant positive correlation ($p < 0.05$) with FCE, while albumin was correlated only with ADG. Triiodothyronin (T_3), a growth moderator, and thyroxin (T_4) showed a negative correlation with ADG, with its optimum

TABLE 4 | Correlation matrix between the different blood parameters, ADG and FCE.

Variables	Urea	Total protein	Albumin	Cholesterol	LDL	HDL	TG	LDH	SGOT	SGPT	Phosphorus	IGF-1	T-3	T-4	ADG	FCE
Urea	**1**	0.1531	−0.0535	0.0495	−0.2274	0.0761	0.0836	−0.3115	−0.0511	0.1148	0.1812	−0.0764	−0.0839	−0.1645	−0.1266	−0.0522
Total protein	0.1531	**1**	**0.6087**	**0.4754**	0.1116	−0.1849	0.3266	**0.3846**	**0.4634**	0.2670	−0.1321	0.0274	−0.1402	−0.3176	−0.0701	**0.4726**
Albumin	−0.0535	**0.6087**	**1**	0.3386	0.0153	−0.0692	0.3302	**0.4834**	**0.3815**	0.2246	0.0302	0.1523	−0.1492	**−0.4111**	**0.3610**	**0.3845**
Cholesterol	0.0495	**0.4754**	0.3386	**1**	0.2852	0.0164	0.2571	0.2837	**0.4533**	0.2687	0.0352	0.0176	−0.1116	**−0.3830**	0.2518	0.2035
LDL	−0.2274	0.1116	0.0153	0.2852	**1**	0.2516	**−0.3822**	−0.0740	0.0114	−0.0972	−0.1579	**0.3600**	−0.0788	0.0475	−0.0089	−0.1762
HDL	0.0761	−0.1849	−0.0692	0.0164	0.2516	**1**	0.2171	−0.2663	0.0140	0.1140	−0.1671	**0.4216**	−0.2664	0.1514	0.0718	−0.2444
TG	0.0836	0.3266	0.3302	0.2571	**−0.3822**	0.2171	**1**	0.2726	**0.3947**	0.3329	0.1378	−0.1410	−0.0374	−0.1962	−0.0223	0.2621
LDH	−0.3115	**0.3846**	**0.4834**	0.2837	−0.0740	−0.2663	0.2726	**1**	**0.4422**	0.2288	−0.2182	−0.0071	−0.0240	−0.3494	0.0221	**0.3938**
SGOT	−0.0511	**0.4634**	**0.3815**	**0.4533**	0.0114	0.0140	**0.3947**	**0.4422**	**1**	**0.7233**	−0.2136	0.2169	0.0470	**−0.3627**	0.0287	**0.4042**
SGPT	0.1148	0.2670	0.2246	0.2687	−0.0972	0.1140	0.3329	0.2288	**0.7233**	**1**	−0.0494	**0.3979**	−0.0843	−0.2316	0.2218	0.1553
Phosphorus	0.1812	−0.1321	0.0302	0.0352	−0.1579	−0.1671	0.1378	−0.2182	−0.2136	−0.0494	**1**	0.0018	0.0786	−0.1030	0.0526	−0.1900
IGF-1	−0.0764	0.0274	0.1523	0.0176	**0.3600**	**0.4216**	−0.1410	−0.0071	0.2169	**0.3979**	0.0018	**1**	−0.2198	−0.1561	0.1430	0.0675
T-3	−0.0839	−0.1402	−0.1492	−0.1116	−0.0788	−0.2664	−0.0374	−0.0240	0.0470	−0.0843	0.0786	−0.2198	**1**	**0.4669**	**−0.4446**	0.1402
T-4	−0.1645	−0.3176	**−0.4111**	**−0.3830**	0.0475	0.1514	−0.1962	−0.3494	**−0.3627**	−0.2316	−0.1030	−0.1561	**0.4669**	**1**	−0.1755	−0.2082
ADG	−0.1266	−0.0701	**0.3610**	0.2518	−0.0089	0.0718	−0.0223	0.0221	0.0287	0.2218	0.0526	0.1430	**−0.4446**	−0.1755	**1**	−0.1810
FCE	−0.0522	**0.4726**	**0.3845**	0.2035	−0.1762	−0.2444	0.2621	**0.3938**	**0.4042**	0.1553	−0.1900	0.0675	0.1402	−0.2082	−0.1810	**1**

Values in bold are different from 0 with a significance level alpha = 0.05.

TABLE 5 | Performance evaluation matrices of machine learning algorithms developed for the prediction of FCE using blood parameters and ADG as predictor variables.

Classifiers	Cross-validation					
	Correlation coefficient (CC)		Mean absolute error (MAE)		Root mean squared error (RMSE)	
	10-fold	Leave one out	10-fold	Leave one out	10-fold	Leave one out
RBF network	0.31	0.48	0.009	0.09	0.12	0.117
Isotonic regression	0.58	0.55	0.089	0.09	0.10	0.115
SMO Reg (RBF kernel)	0.23	0.16	0.100	0.10	0.13	0.130
IBK	0.30	0.29	0.108	0.11	0.14	0.139
LWL	0.53	0.47	0.09	0.09	0.11	0.118
RF	0.35	0.27	0.09	0.10	0.12	0.127
Additive regression (isotonic regression)	***0.60***	***0.45***	***0.09***	***0.11***	***0.12***	***0.150***

Bold values indicates the significant.

TABLE 6 | Goodness-of-fit statistics for the three PLSR models.

Goodness-of-fit statistics over different models (variable FCE)			
Observations	**All FCE**	**Low FCE (positive RFI)**	**High FCE (negative RFI)**
	31.0000	16.0000	15.0000
Sum of weights	31.0000	16.0000	15.0000
DF	28.0000	13.0000	12.0000
$\boldsymbol{R^2}$	**0.7638**	**0.9068**	**0.9423**
Std. deviation	0.0671	0.0220	0.0262
MSE	0.0041	0.0004	0.0005
RMSE	0.0637	0.0198	0.0234

Where, DF= the no. of degrees of freedom of the selected model.
$\boldsymbol{R^2}$ *= coefficient of determination, interpreted as proportion of variability of the dependent variable as explained by the model.*
Bold values indicates the significant.

level in circulation in efficiently feed-utilizing heifers, which corroborates with an earlier study (10). A significant positive correlation of LDH and SGOT with FCE indicates that feed utilization is an energy-dependent function, which requires higher reducing power for optimum productivity of the animal. IGF 1 and its interaction with the other blood parameters were observed to be highly influential in high FCE animals, corroborating with earlier findings (52).

Physiological Model

The objective of developing the **machine learning** model was to capture the dynamics of blood parameters and ADG in relation to FCE to establish performance evaluation matrices of the tested machine learning algorithms on 10-fold cross-validation and leave-one-out cross-validation for the feed conversion efficiency trait in heifers (**Table 5**).

Isotonic regression and LWL (locally weighted learning) performed better than all other machine learning algorithms, when blood parameters and ADG were used as predictor variables for FCE (response variable) (**Table 5**). The performance of isotonic regression is further increased by using it as a base classifier with a meta-classifier (additive regression).

We further ranked the blood parameter variables as per their importance in respect of predicting FCE, using the Relieff feature ranking algorithm (53). The Relieff algorithm works by assigning higher and lower weights to the different predictor variables based on their importance in predicting the response variable. The order of ranking in respect of predictor variables, i.e., blood parameters in relation to FCE, as response variable using Relieff algorithm, was urea >TP >albumin >cholesterol >5-LDL >HDL >triglyceride >LDH >SGOT >SGPT >phosphorus >IGF-1 >T3 >T4 >ADG in the present study.

Further, to understand the possible higher or lower interactions between the dependent and independent variables for getting insights into the possible physiological indicators of FCE, PLSR models (**Figure 1**) were developed as a more descriptive modeling technique. Three separate models were developed using blood parameters and ADG as the independent variable and FCE (RFI) values as the dependent variable, considering all positive and negative RFI values as model (i), only higher FCE (negative RFI) values as model (ii), and only lower FCE (positive RFI) as model (iii), respectively. Two components in the model gave better evaluation indices for the three separate models. The best PLSR model obtained was that for higher FCE (negative RFI) values (**Figure 1B**). The quality of the model was evaluated on the basis of Q^2-cumulated (Q^2 cum), R^2Y-cumulated (R^2Y cum), and R^2X-cumulated (R^2X cum) values (**Figure 1**); two component (new predictor variables which are constructed using the linear combinations of the original predictor variables, also known as latent variables) models are better than one-component model. The higher the Q^2 cum, R^2Y cum, and R^2X cum, the better is the quality of the model.

As per the goodness-of-fit statistic, the coefficient of determination (R^2) values of the model considering all FCE values is least depicting as compared to the other two models (**Table 6**). The PLSR model for the higher FCE values was the best based on performance evaluation metrics (R^2 **0.90680**) over lower FCE (R^2 0.9423) and all FCE ($\boldsymbol{R^2}$ **0.7638**) in the present study, based on the criteria of the qualifying PLSR model having $R^2 > 0.7$ and $Q^2 > 0.4$ (54). For all the three PLSR models, plots of observed and predicted values are shown in **Figure 2**. Most of the predicted values were recorded within a 95% confidence interval.

This study indicates that variation in blood parameters and metabolic characteristics, if it does not lead to a more efficient and early selection, at least would be useful for selective genotyping for RFI/FCE through identified physiological and genetic markers.

The two horizontal lines on the VIP bar charts (**Figure 3**) represent the two thresholds at 0.8 and 1. The variables having moderate influence depicted as VIP score between >0.8 & <1. Those highly influential variables have a VIP score >1.

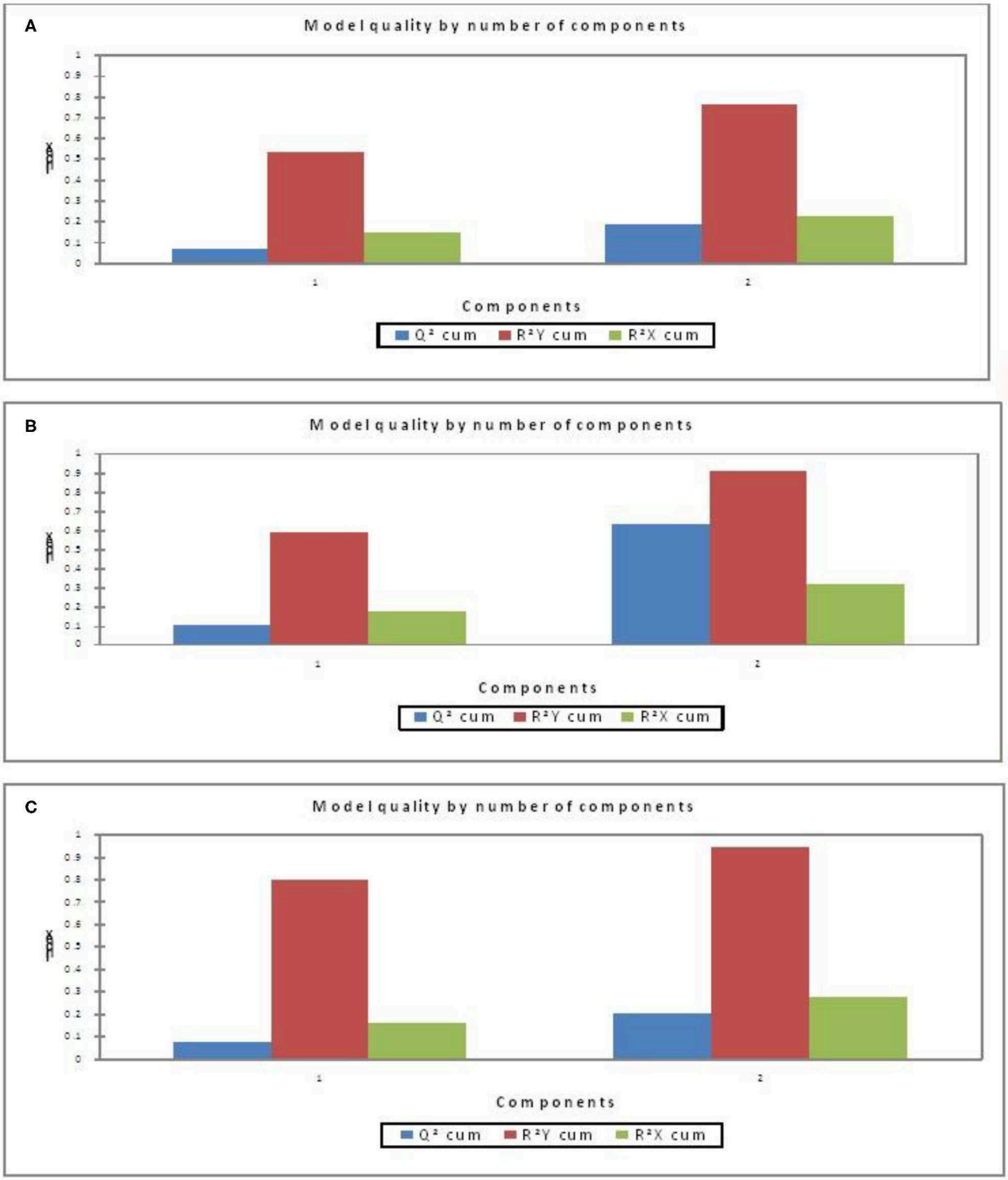

FIGURE 1 | Model quality by number of components **(A)** all FCE, **(B)** higher FCE, **(C)** lower FCE).

VIP-values >0.8 are significant (55) for blood parameters and ADG which differ in respect of all the three PLSR models. In the higher FCE group, IGF1 and its interaction were highly influential, while in the lower FCE group, albumin and its interaction were more influential (**Table 7**). IGF1 is known to regulate the levels of blood glucose, mostly (up to 90%) by

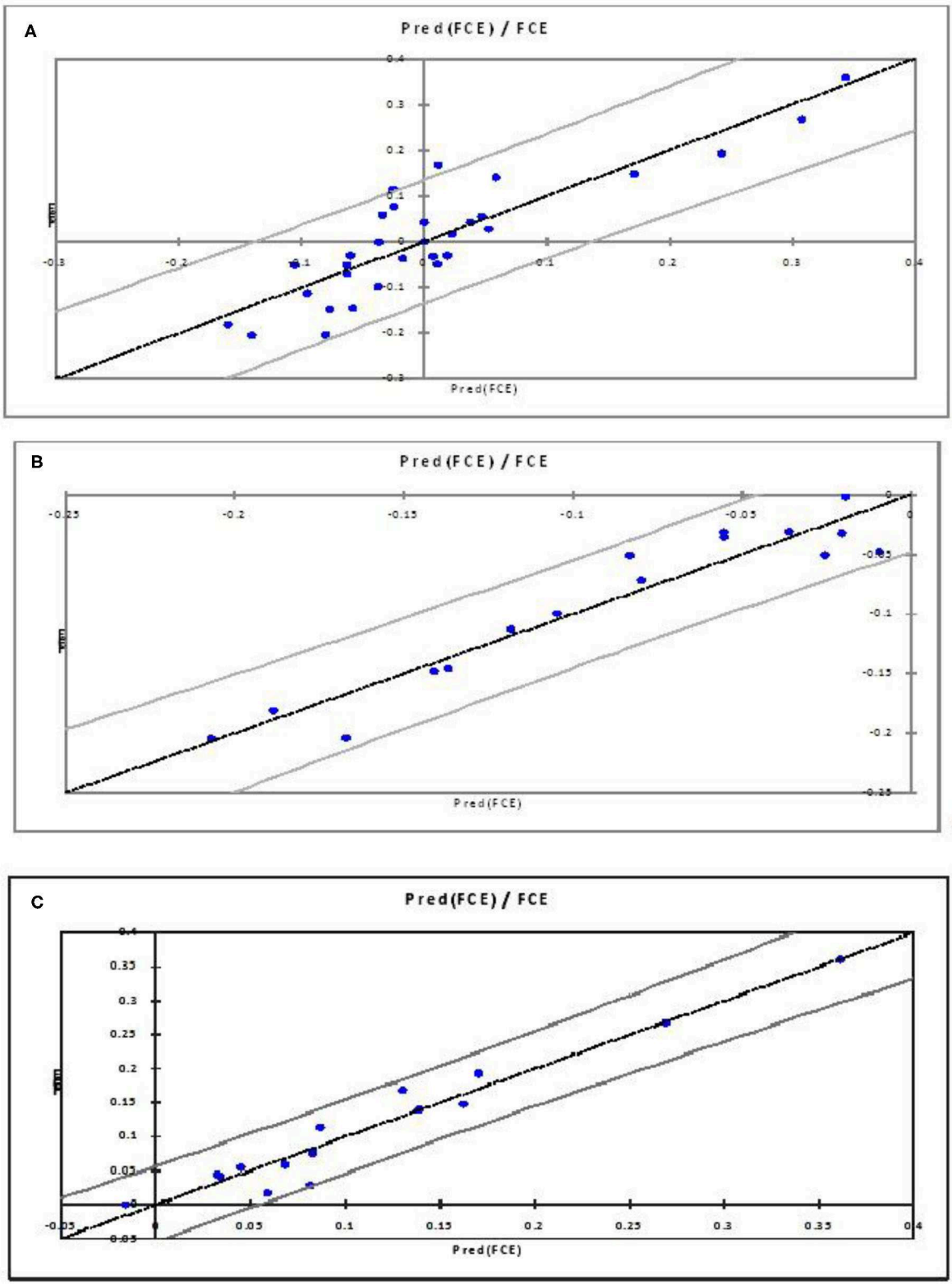

FIGURE 2 | Observed and predicted FCE values for All FCE **(A)**, higher FCE/negative RFI **(B)**, and lower FCE/positive RFI **(C)**.

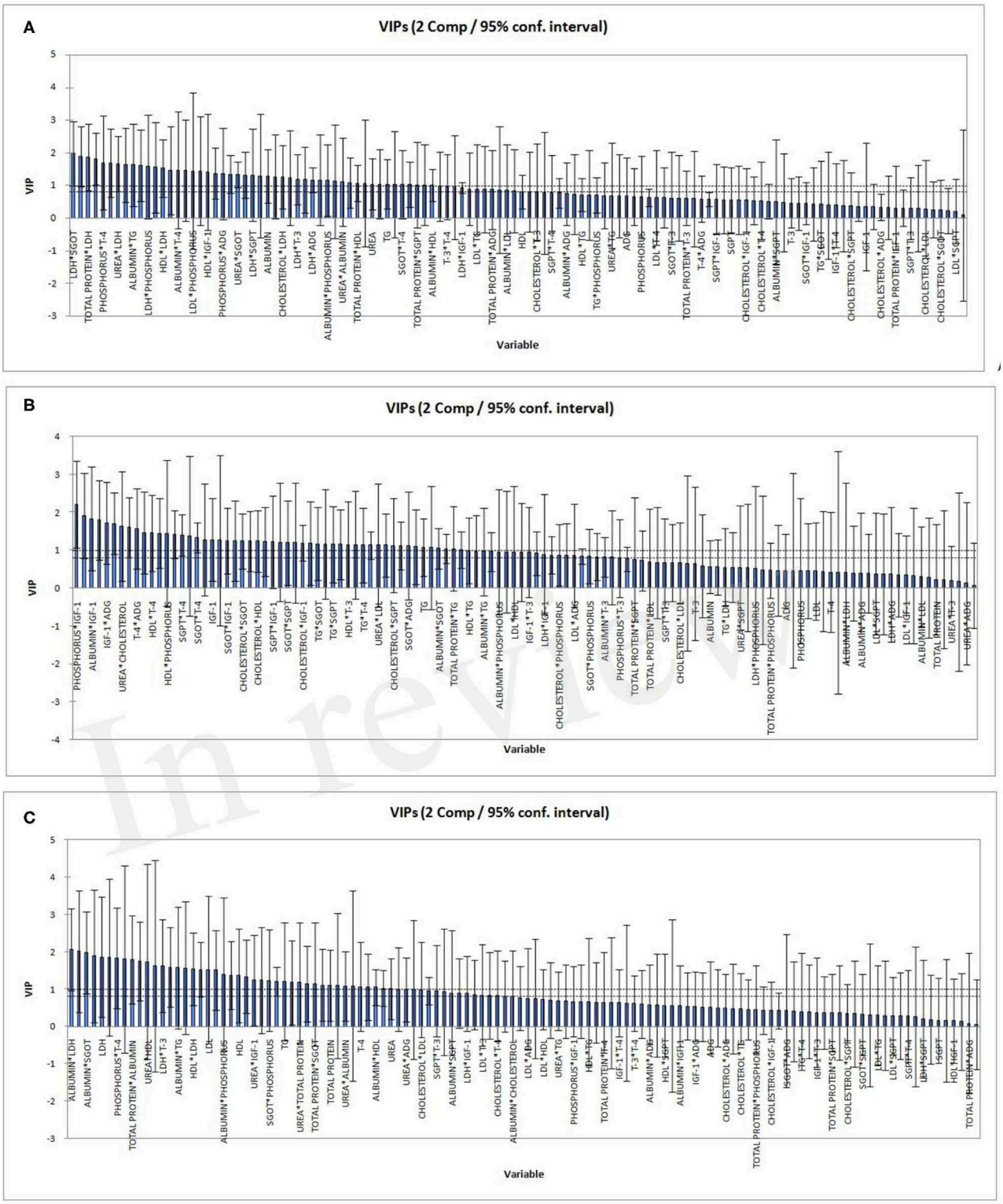

FIGURE 3 | Variable importance for the projection (VIP) for the two components for the three PLSR models: all FCE **(A)**; high FCE **(B)**; low FCE **(C)**.

In review

TABLE 7 | Significant interactions among blood metabolic indicators based on VIP charts emerged from PLSR models.

Blood attribute interactions		
Using all RFI	**Negative RFI/higher FCE subgroup**	**Positive RFI/lower FCE subgroup**
LDH*SGOT	Phosphorus*IGF-1	Albumin*LDH
TP*LDH	Albumin*IGF1	Albumin*SGOT
Phosphorus*T4	IGF1*ADG	LDH
Urea*LDH	Urea*Cholesterol	Phosphorus*T4
Albumin*TG	T4*ADG	TP*Albumin
LDH*Phosphorus	HDL*T4	Urea*HDL
HDL*IFG-1	HDL*Phosphorus	LDH*T3
Phosphorus*ADG	SGPT*T4	Albumin*T4
Urea*SGOT	SGOT*T4	HDL*LDH
LDH*SGPT	IGF1	LDL
Albumin	SGOT*IGF1	Albumin*Phosphorus
Cholesterol*LDH	Cholesterol*SGOT	HDL
LDH*T3	Cholresterol*HDL	UREA*IGF1
LDH*AFG	SGPT*IGF1	SGOT*Phosphorus
Albumin*Phosphorus	SGOT*SGPT	TG
Urea*Albumin	Cholesterol*IGF1	Urea*TP
TP*HDL	TG*SGOT	TP*SGOT
Urea	TG*SGPT	TP
Triglyceride	HDL*T3	Urea*Albumin
SGOT*T4	TG*T4	T4
TP*SGPT	Urea*LDL	Albumin*HDL
Albumin*HDL	Cholesterol*SGPT	Urea
T3*T4	SGOT*ADG	Urea*ADG
	Triglyceride	
	Albumin*SGOT	
	TP*TG	
	HDL*TG	
	Albumin*TG	
	Albumin*Phosphorus	

gluconeogenesis, using non-carbohydrate entities as amino acid metabolism (28, 56). This study reveals the relation between IGF1 and FCE in growing young female buffalo calves corroborating with earlier reports (57).

CONCLUSIONS

Blood parameters depicting intermediary metabolism were recorded for buffalo heifers, maintained at the Govt. Livestock farm. Their interactive influence along with ADG over FCE has been established using the machine learning approach in the present study. Blood analyses are known to reflect the status of energy metabolism and some attributes were related to feed efficiency of heifers.

We developed machine learning models using blood parameters and ADG as the predictor variable and FCE as the response variable. PLSR models were developed separately for all animals, only efficient (negative RFI), and inefficient animals (positive RFI), to facilitate understanding of blood parameter interaction with ADG and FCE. The machine learning model based on isotonic regression outperformed other machine learning algorithms used for modeling in the present study. Further, the predictive accuracy of isotonic regression was enhanced using additive regression. The developed machine learning models are found effective in predicting FCE accurately. Further, the ranking of predictor variables was evaluated to predict FCE. It may facilitate understanding of intricate dynamics of blood parameters underlying growth.

As deduced from the VIP charts of PLSR, FCE is affected by IGF1 and its interactions with other blood parameters in the higher FCE group. IGF1 regulates the blood glucose level, amino acid metabolism, and protein synthesis. IGF1 has also been found related with FCE in growing heifers.

The predictive accuracy of the machine learning models can be further increased by the inclusion of a broader range of blood parameters, which can then be used as a phenotypic marker for selection of efficient animals. To the best of our knowledge, this is the first report of modeling of blood attributes and ADG with FCE in *Bubalus bubalis*. Our study is the first to show that a machine learning predictive model based on blood tests alone can be successfully applied to predict FCE in heifers and could open up unprecedented possibilities in feed trial-based cumbersome diagnosis.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because it is a property of Indian Council of Agricultural Research, New Delhi. Requests to access the datasets should be directed to www.cirb.res.in.

ETHICS STATEMENT

The animal study was reviewed and approved by Institute Animal Ethics Committee of CIRB Hisar.

AUTHOR CONTRIBUTIONS

PS: design and execution of experiment and developed the manuscript. AN: developed the machine learning models. SP: performed the experiment. JA and AB: blood analysis. DM and KC: statistical analysis. AR: manuscript. KY: data collection and handling. SB: data analysis. All authors: contributed to the article and approved the submitted version.

FUNDING

This study was supported by a grant from ICAR Network Project on Agricultural Bioinformatics (CAB) in scheme, IASRI, New Delhi.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 08 September 2020
doi: 10.3389/fvets.2020.00622

Seasonal Dynamics of Physiological, Oxidative and Metabolic Responses in Non-lactating Nili-Ravi Buffaloes Under Hot and Humid Climate

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Edited by:
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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: 16 April 2020
Accepted: 31 July 2020
Published: 08 September 2020

Citation:
Li M, Hassan F, Guo Y, Tang Z, Liang X, Xie F, Peng L and Yang C (2020) Seasonal Dynamics of Physiological, Oxidative and Metabolic Responses in Non-lactating Nili-Ravi Buffaloes Under Hot and Humid Climate. Front. Vet. Sci. 7:622. doi: 10.3389/fvets.2020.00622

Hot and humid weather exposes animals to high temperature and relative humidity that ultimately reduce their ability to disperse body heat. To avoid serious consequences of heat stress, it is imperative to understand animal physiological responses and biochemical changes during a state of altered body homeostasis across different seasons of the year. This study evaluated seasonal dynamics of physiological, oxidative, and metabolic responses of Nili-Ravi buffaloes to hot and humid climate. Twenty non-lactating multiparous buffaloes were enrolled for this 1-year study. Meteorological data were recorded twice daily to calculate temperature humidity index (THI). Physiological parameters including rectal temperature (RT), body surface temperature (BST), and respiratory rate (RR) were measured weekly. Blood samples were collected once in each season (spring, summer, autumn, and winter) to analyze biochemical and antioxidant parameters. We also measured activities of liver enzymes including alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The results revealed a significantly higher THI value (82) during summer which resulted in a significant increase in RR and BST as compared to winter. Higher oxidative stress was observed in summer owing to significantly higher malondialdehyde (MDA) content and lower levels of serum antioxidant enzymes (GPx, SOD, and CAT) as compared to other seasons. Moreover, serum cortisol was also significantly higher while adrenocorticotropic hormone (ACTH), Triiodothyronine (T_3), insulin, and growth hormone contents were significantly lower in summer. Contrarily, plasma thyroxin (T_4) level was higher in summer. THI showed a positive correlation with physiological responses but a negative correlation with antioxidant parameters. Our study provides practical insights on the adaptive physiology of buffaloes and has several implications regarding the alleviation of heat stress in buffaloes to enhance the efficiency of production and reproduction under tropical climate. Our study suggests the use of appropriate cooling strategies to effectively manage the non-lactating buffaloes to avoid performance losses and animal welfare issues in summer season.

Keywords: temperature humidity index, heat stress, physiological response, oxidative stress, serum metabolites, buffalo

INTRODUCTION

Climate change imposes adverse effects on animal physiology, leading to an overall decrease in efficiency of production and reproduction. Moreover, it also raises animal health and welfare concerns, sometimes with serious consequences. Heat stress is one of the major climatic effects faced by animals, especially in tropical and sub-tropical countries. Heat stress in the tropics is generally associated with animal welfare issues and significant economic losses resulting from reduced performance but increased morbidity and mortality of livestock (1). The impact of heat stress is expected to become worse in the recent climate change scenario as it will increase the potential intensity of hot and humid conditions in the future, leading to an increased frequency of heat stress episodes (2). At higher ambient temperatures (above 30°C), excessive heat load hampers the ability of animals to dissipate their body heat. Heat stress occurs usually when animals are unable to maintain the balance between heat produced/stored and heat dissipated (3, 4). To manage this excessive heat load, animals attempt to reduce metabolic heat production while increasing heat dissipation to maintain euthermia. This is accompanied by a series of physiological, metabolic, and behavioral manifestations to thrive and mitigate adverse effects of heat stress. The first line of response to excessive heat load mainly includes accelerated respiratory rate (RR), increased water intake but reduced feed intake (5). Physiological parameters like body surface temperature (BST), rectal temperature (RT), respiratory rate, and pulse rate (PR) are the quick, ultimate responses of animals to climatic stress and eventually the level of discomfort or comfort of animals in a given environment (6).

Heat stress exposes dairy animals, especially buffaloes, to an excessive load of reactive oxygen species (ROS), leading to severe oxidative stress that subsequently reduces metabolic activity and immune response (7). Antioxidant system of animals fails to scavenge a large number of free radicals in time due to reduced activities of antioxidant enzymes (CAT, SOD, and GPx) under chronic heat stress (8). Therefore, levels of oxidative and antioxidant enzymes in ruminants can be used as markers to assess the degree of oxidative stress (9). Exposure to high ambient temperature also adversely affects the activity of the thyroid gland owing to its high sensitivity to environmental heat variations, subsequently leading to reduced levels of T_3 and T_4 (10). This decline in thyroid activity is coupled with a decrease in growth hormone to reduce metabolic activity and body heat increment. Moreover, stimulation of the hypothalamus pituitary–adrenal axis leads to the secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland and then triggers the synthesis and secretion of glucocorticoids like cortisol (11). Hence, serum cortisol has been used extensively as a biomarker of heat stress in cattle (12, 13) and buffalo (14, 15).

These physiological manifestations in response to heat stress, significantly affect biological rhythm (diurnal and seasonal) in dairy animals. Compared to Zebu cattle, these effects are more pronounced in buffaloes owing to their dark skin color and lack of sweat glands. Heat stress triggers many physiological changes in the body of buffaloes, including a decrease in feed intake, shift in hormonal and metabolic secretions but an increase in BST, RT, RR, and oxidative stress (16, 17). Thermal comfort is generally measured by temperature-humidity index (THI) and has been applied to different livestock species, to understand the physiological stages of thermo-neutral and heat stress zones. It is imperative to better understand the changes in physiological and metabolic rhythms in buffaloes during different seasons, to devise strategies to mitigate the adverse effects of heat stress so that to avoid economic losses and animal welfare issues. Moreover, the association of THI with various physiological and biochemical parameters in buffaloes is a precondition to predict environmental effects during different seasons of the year. It is pertinent to mention that tropical climate of Southern China is quite different from other South Asian countries due to quite higher humidity which substantially increases average THI. Moreover, to best of our knowledge, no study is available regarding the seasonal variations in the oxidative physiology of non-lactating buffaloes under hot and humid climate of south China. Therefore, this study was planned to explore variations in biological rhythms of physiological, metabolic, hormonal, and oxidative responses of non-lactating Nili-Ravi buffaloes to tropical climatic conditions in Southern China.

MATERIALS AND METHODS

Animal Welfare Statement

All procedures used in this experiment were approved by the Ethics Committee of the Guangxi Buffalo Research Institute, Chinese Academy of Agriculture Sciences China (Approval No. BRI2017006). All steps of animal rearing, recording of physiological parameters and blood sample collection were conducted strictly according to the guidelines of the committee. We confirm that we followed the rules of the Declaration of Helsinki for recording biological rhythms in buffaloes during this experiment.

Geographical Location and Climate

This study was conducted at the Guangxi Buffalo Research Institute (22°53′22.59″ N; 108°21′51.19″ E; 122 m above sea level) located in Nanning, China. The overall climate of this area is characterized as hot and humid as it is located in the subtropical monsoon climate zone. This area receives sufficient sunshine with abundant rainfall (average 1,304 mm) with longer summer and short winter seasons. We used an online dust monitoring system (Shenzhen Greenford Environmental Technology Co., Ltd.) to record weather data in real-time, mainly including air temperature (AT, °C) and relative humidity (RH, %), with an interval at 30 min and at an installation height of buffalo's body.

Environmental variables were recorded twice daily in the morning (at 8:00 A.M.) and in the afternoon (at 2:30 P.M.) and used to depict monthly meteorological variations during the whole study period (**Figure 1**). Average daily temperature and relative humidity were recorded during the study period from April 2017 to March 2018 (for 1 year) and Temperature Humidity Index (THI) was calculated by using the following formula (18):

$$\mathrm{THI} = \mathrm{AT} + 0.36\ \mathrm{DPT} + 41.5$$

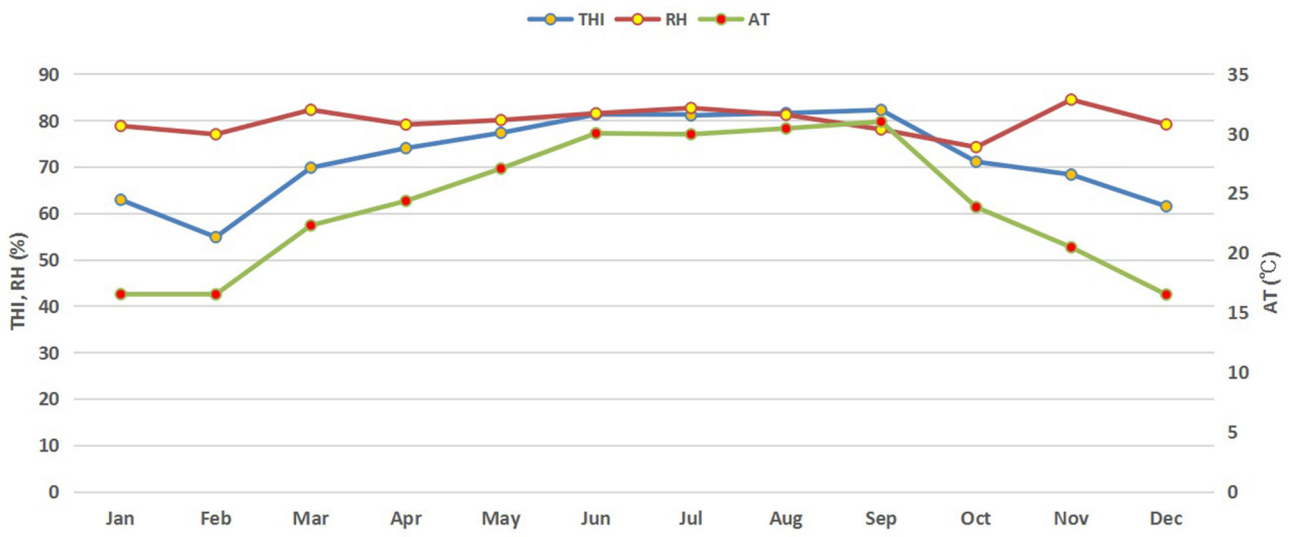

FIGURE 1 | Monthly trends of THI, AT, and RH during the study period. THI, temperature humidity index; RH, relative humidity (%); AT, air temperature (°C).

Where, AT is the air temperature and DPT the dew point temperature of the buffalo shed. Four seasons of the year were defined as; spring (March–May), summer (June–August), autumn (September–November), and winter (December–February).

Selection and Management of Buffaloes

Twenty non-lactating Nili-Ravi buffaloes were randomly selected for this experiment. All buffaloes were multiparous (3–5 lactations) with an average body weight of 550 ± 50 Kg. Buffaloes were housed in an open uncovered area with a stocking density of 15 m^2/head throughout the study period. Free access to water was provided to all buffaloes throughout the day. Mist spraying was performed on buffaloes whenever the temperature exceeded 28°C throughout the year. All buffaloes were fed a total mix ration (TMR) consisting of grass (*Pennisetum purpureum schum*), corn silage and concentrate mixture (corn, soybean meal, and wheat bran). The same quantity of TMR was fed daily to each animal to meet its dietary requirement as per the general routine of the buffalo farm. The formulation and chemical composition of total mix ration are presented in **Table 1**.

TABLE 1 | Feed composition and nutrient levels of the diet (air-dry basis,%).

Ingredient	Content (%)
Grass (*Pennisetum purpureum schum*)	29.6
Silage corn	48.2
Corn	16
Soybean meal	2
Wheat bran	1
Lime stone	0.5
$CaHPO_4$	0.5
$NaHCO_3$	0.7
NaCl	0.5
Premix[a]	1
Total	100
Nutrient level[b]	
CP	8.96
NDF	46.34
ADF	25.68
Ash	5.65

[a] *The additive premix provided the following elements per Kg of diets: Vitamin A 550,000 IU, Vitamin E 3,000 IU, Vitamin D3 150,000 IU, Fe (as ferrous sulfate) 4.0 g, Cu (as copper sulfate) 1.3 g, Mn (as manganese sulfate) 3.0 g, Zn (as zinc sulfate) 6.0 g, and Co (as cobalt sulfate) 80 mg.*
[b] *Measured values.*

Recording of Physiological Parameters and Blood Sampling

Physiological parameters of buffaloes were recorded weekly as follows:

Body Surface Temperature (BST)

Average BST was recorded each Tuesday at 8:00 A.M. and 2:30 P.M. using an animal infrared thermometer from three different body sites, including forehead, left chest, and abdomen. Three respective values were averaged to get weekly BST for each buffalo.

Rectal Temperature (RT)

At the same time, RT was also recorded by inserting a veterinary rectal thermometer in the rectum of buffalo for 2 min.

Respiratory Rate (RR)

Weekly RR was recorded as times/min by observing and counting thoracic movements using a stopwatch and a counter for 2 min.

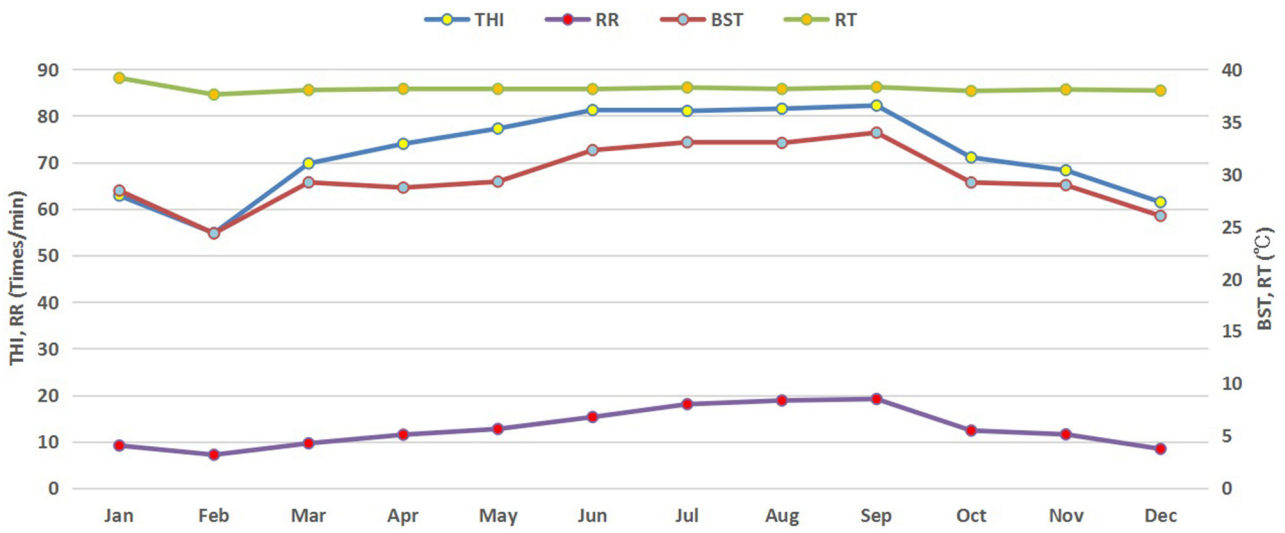

FIGURE 2 | Monthly trends of physiological parameters of buffaloes in response to THI. THI, temperature humidity index; RT, rectal temperature (°C); RR, respiratory rate (times/minute); BST, body surface temperature (°C).

Blood Sampling and Determination of Biochemical Parameters

Blood samples (10 mL) from each buffalo were collected from jugular vein before morning feeding once during each season viz: spring (April 17th), summer (July 22nd), autumn (October 18th), and winter (January 28th). These samples were used to determine serum hormones and antioxidant enzymes as follows:

Serum Hormones

Blood samples were put on ice after the collection and immediately transferred to the laboratory for serum isolation. Blood samples were centrifuged at 3,000 rpm for 15 min, and serum was harvested according to standard methods (19). Isolated serum was stored at −20°C until further analysis. Serum hormones including Adrenocorticotropic hormone (ACTH), Insulin, Cortisol, Triiodothyronine (T_3), Thyroxine (T_4), and growth hormone (GH) were analyzed using commercial ELISA kits according to manufacturer's instructions (CUSABIO BIOTECH CO., Wuhan, China). The coefficient of variation (inter and intra-assay) of these kits was <10% for each assay.

Serum Antioxidant Enzymes

Concentrations of serum antioxidant enzymes including total antioxidant capacity (TAC), malondialdehyde (MDA), superoxide dismutase (SOD), Glutathione peroxidase (GPx), and catalase (CAT) were determined through spectrophotometer using the Nanjing Built-in Kits (www.njjcbio.com) according to manufacturer's instructions. Average values for each enzyme in each season for all buffaloes are expressed as Mean ± s.e. The coefficient of variations (inter and intra-assay) of these kits was in the same range (<10%).

Plasma Biochemical Parameters

Plasma biochemical parameters including total protein (TP), albumin (ALB), globulin (GLB), blood urea nitrogen, and glucose were determined using respective commercially available kits according to manufacturer's instructions. Activities of liver enzymes ALT and AST were also determined using standard kits according to manufacturer's instructions.

Statistical Analysis

Data were analyzed using the general linear model by analysis of variance in SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). Pearson correlation coefficients between THI and different physiological and biochemical parameters were calculated using the CORR command in SAS.

RESULTS

Monthly Trend of Meteorological Data

Monthly variations in THI, ambient temperature and relative humidity of the buffalo house are presented in **Figure 1**. Higher average THI values (above 80) were observed in summer (June–September) but started to decrease in autumn and winter. It started increasing again in spring. Similar trends in RR and BST of lactating buffaloes were also observed (**Figure 2**).

Effect of THI on Physiological Parameters in Different Seasons

The average values of THI and physiological parameters of non-lactating buffaloes in different seasons are provided in **Table 2**. The THI indices in spring, summer, and autumn were significantly higher than that in winter ($p < 0.05$), with the highest value in summer, followed by autumn and spring. Moreover, RR, BST, and RT were all the highest in summer, while RT did not change across different seasons ($p < 0.05$).

TABLE 2 | THI and physiological indices of non-lactating buffaloes in different seasons.

Parameters	Spring	Summer	Autumn	Winter
Temperature humidity index (THI)	74.49 ± 4.38^{a}	82.00 ± 0.53^{a}	75.51 ± 6.86^{a}	60.25 ± 4.73^{b}
Respiratory rate (times/min)	13.27 ± 1.72^{ab}	17.32 ± 1.09^{a}	15.16 ± 2.82^{ab}	10.61 ± 3.14^{b}
Body surface temperature (°C)	29.53 ± 0.35^{b}	33.17 ± 0.52^{a}	31.37 ± 2.50^{ab}	25.96 ± 1.25^{c}
Rectal temperature (°C)	38.11 ± 0.11	38.26 ± 0.04	38.18 ± 0.16	38.04 ± 0.06

Different superscripts in same row indicate significant difference at $p < 0.05$.

Effect of Season on Serum Antioxidant Enzymes

MDA concentrations were significantly ($p < 0.05$) higher in summer and autumn than in winter and spring (**Figure 3**). TAC was significantly higher in summer than in other seasons ($p < 0.05$) while the differences between spring, autumn, and winter were not significant ($p > 0.05$). However, serum concentrations of GSH, SOD, and CAT were significantly lower in summer but significantly higher in winter compared to other seasons ($p < 0.05$) as presented in **Figure 3**.

Effect of Season on Serum Hormones

Significantly higher ACTH was observed in winter than other seasons ($p < 0.05$) (**Table 3**). However, cortisol was significantly higher in summer than in other seasons ($p < 0.05$). The insulin concentration in summer was significantly lower than other seasons ($p < 0.05$). Furthermore, GH and T_3 concentrations were significantly lower in summer and autumn as compared to spring and winter ($p < 0.05$). Contrarily, T_4 concentration was significantly higher in summer and autumn than in spring and winter ($p < 0.05$).

Effect of Season on Serum Biochemical Parameters

Total protein contents were higher in summer followed by spring, autumn, and winter while albumin was significantly higher ($p < 0.05$) in winter than other seasons (**Table 4**). Globulin was significantly higher in summer than spring and autumn followed by winter at the end ($p < 0.05$). Alanine aminotransferase (ALT) enzyme activity was significantly higher in summer than in other seasons ($p < 0.05$). Aspartate aminotransferase (AST) enzyme activity was significantly higher in spring than in other seasons ($p < 0.05$). Urea nitrogen was significantly lower in autumn than in other seasons ($p < 0.05$). Glucose was significantly higher in winter and autumn followed by summer and then winter at the end ($p < 0.05$) (**Table 4**).

Correlation of THI With Physiological and Biochemical Parameters

At significant levels ($p < 0.05$ or 0.01), Pearson correlation analysis showed positive associations of THI with all three physiological parameters of the non-lactating buffaloes, while RT, BST, and RR also positively correlated between each other. Negative correlations ($p < 0.05$) of THI with ACTH and cortisol were also observed. Both cortisol and T_4 were positively correlated each other first and then with all three physiological parameters. Cortisol was negatively correlated with ACTH while T_3 showed a positive correlation with GH (**Table 5**).

Correlation of THI With Serum Antioxidant Enzymes

THI showed highly negative correlations ($p < 0.05$ or 0.01) with GPx, SOD, and CAT but a positive correlation ($P < 0.05$) with TAC in the non-lactating buffaloes (**Table 6**). TAC content showed negative correlations ($p < 0.05$ or 0.01) with GPx and SOD and MDA content was negatively associated with GPx and CAT ($p < 0.01$). However, positive collections ($P < 0.05$ or 0.01) were observed between GPx, SOD, and CAT (**Table 6**).

DISCUSSION

Climate is one of the major factors that cause oxidative stress and challenge the defense system of an animal, subsequently leading to many physiological manifestations. These physiological changes are the immediate responses of an animal to climatic stress and eventually reflect the level of discomfort or comfort of an animal in a given environment (6). The most obvious physiological effects of heat stress on animals include pronounced changes in their heart and respiratory rates. This metabolic and physiological load induced by heat stress also significantly affects their diurnal and seasonal rhythms (1).

Effect of THI on Physiological Parameters

Our study revealed significant increases in BST and RR of non-lactating buffaloes in summer when THI value went up to 82. Increased RR and pulse rate (PR) reflected the response of animals, as an adaptive strategy, to lower down their body temperatures through evaporative cooling. Studies have reported higher RR and RT but lower PR in buffaloes exposed to high temperatures in a climatic chamber (3) and under pasture grazing system (20). Similarly, dramatic increases in RR, RT, and PR have been observed in cattle under heat stress conditions (21). However, average RT showed no significant difference among different seasons in the present study. Similar findings have been reported in swamp buffaloes at an ambient temperature of 30°C (22, 23). Exposure to high ambient temperatures (>30°C) has shown to stimulate a remarkable increase in RR and subsequent the initiation of panting without any change in RT in buffaloes (22). In our study, despite the high THI in summer, no severe

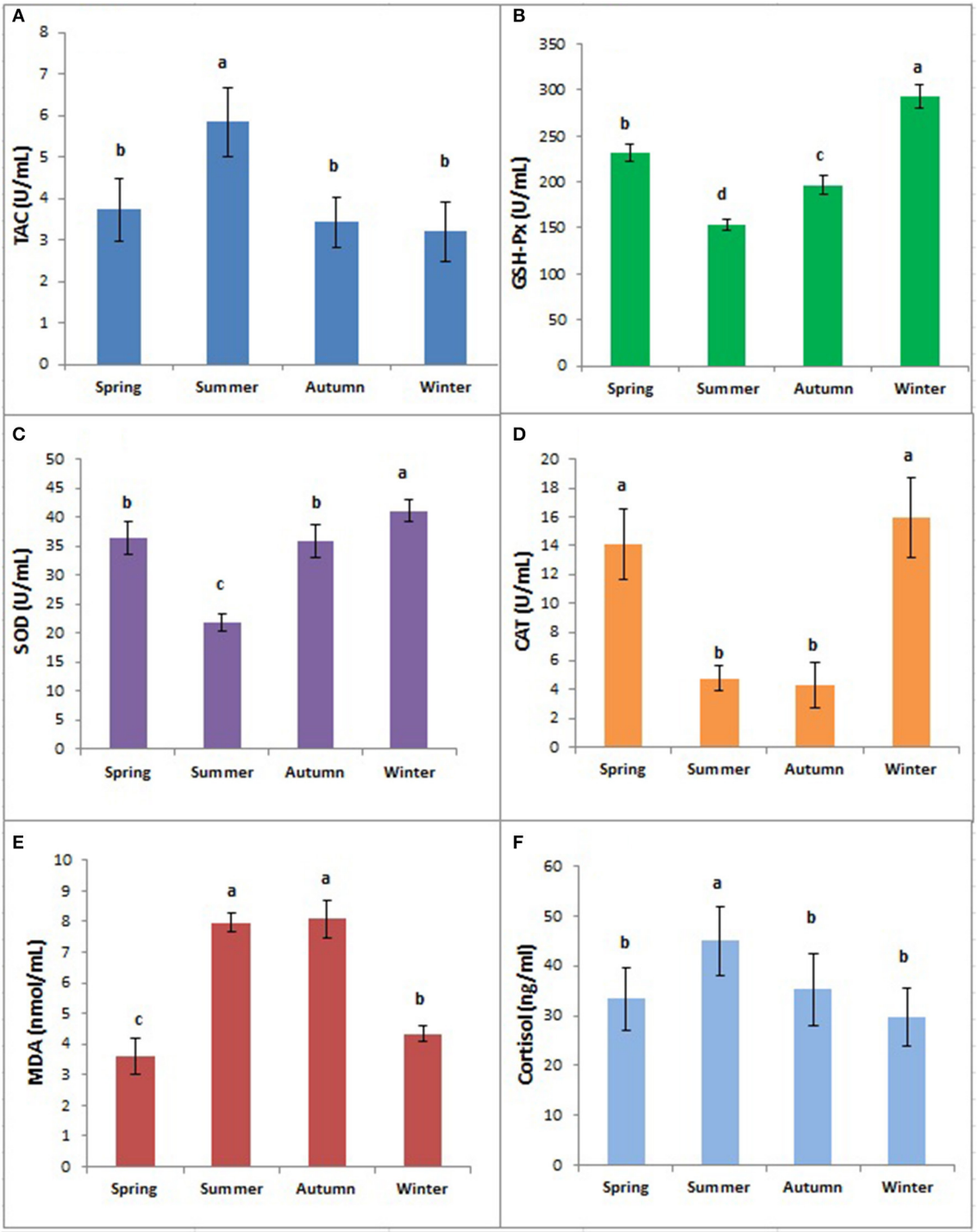

FIGURE 3 | Concentrations of TAC **(A)**, GPx **(B)**, SOD **(C)**, CAT **(D)**, MDA **(E)**, and Cortisol **(F)** in buffaloes in different seasons. Bars marked with different letters differ significantly ($p < 0.05$). TAC, total antioxidant capacity; GPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde.

response was found in physiological parameters of the non-lactating buffaloes, including RT as an index of heat storage in the body. This shows that thermo-tolerant ability of the non-lactating buffaloes along with management practices like mist spraying facilitated the mitigation of adverse effects of heat stress under tropical weather.

TABLE 3 | Seasonal changes in serum hormone concentrations of non-lactating buffaloes.

Items	Spring	Summer	Autumn	Winter
Corticotropin (ACTH) (pg/ml)	84.31 ± 9.52[b]	55.44 ± 11.29[c]	84.94 ± 16.42[b]	101.21 ± 14.81[a]
Insulin (nIU/ml)	11.50 ± 1.64[a]	8.71 ± 2.24[c]	11.11 ± 2.18[ab]	9.89 ± 2.04[bc]
Triiodothyronine (T_3) (ng/ml)	1.57 ± 0.61[a]	0.93 ± 0.34[b]	1.05 ± 0.26[b]	1.33 ± 0.21[a]
Thyroxine (T_4) (ng/ml)	45.43 ± 9.23[b]	53.11 ± 13.70[a]	55.96 ± 8.03[a]	42.12 ± 4.42[b]
Growth hormone (ng/ml)	13.28 ± 2.21[a]	9.52 ± 3.84[b]	9.93 ± 3.25[b]	13.21 ± 4.39[a]

Different superscripts in same row indicate significant difference at $p < 0.05$.

TABLE 4 | Blood biochemical parameters of non-lactating buffaloes during different seasons.

Parameter	Spring	Summer	Autumn	Winter
Total protein (g/L)	79.46 ± 5.78[ab]	82.36 ± 2.87[a]	77.18 ± 2.91[bc]	74.15 ± 5.59[c]
Albumin (g/L)	35.06 ± 3.10[b]	34.49 ± 1.79[b]	34.03 ± 2.06[b]	38.63 ± 2.41[a]
Globulin (g/L)	44.40 ± 5.23[b]	47.87 ± 2.65[a]	43.14 ± 2.45[b]	34.08 ± 3.83[c]
Alanine aminotransferase (U/L)	36.36 ± 9.34[b]	35.41 ± 6.43[b]	44.18 ± 3.40[a]	36.92 ± 5.58[b]
Aspartate aminotransferase (U/L)	125.18 ± 17.23[a]	108.91 ± 13.08[b]	112.81 ± 10.92[b]	107.14 ± 9.70[b]
Blood urea nitrogen (mmol/L)	6.32 ± 1.56[a]	7.00 ± 1.01[a]	5.16 ± 0.83[b]	6.44 ± 0.71[a]
Glucose (mmol/L)	2.57 ± 0.27[c]	3.46 ± 0.18[b]	3.73 ± 0.20[a]	3.92 ± 0.35[a]

Different superscripts in same row indicate significant difference at $p < 0.05$.

TABLE 5 | Correlations of THI with physiological and biochemical parameters of buffaloes.

Item	THI	BST	RT	RR	ACTH	Cortisol	Insulin	T_3	T_4	GH
THI	1									
BST	0.938**	1								
RT	0.849**	0.846**	1							
RR	0.917**	0.951**	0.767**	1						
ACTH	−0.629*	−0.405	−0.118		1					
Cortisol	−0.624*	0.865**	0.833**	0.905**	−0.783**	1				
Insulin	−0.005	0.207	0.515	0.19	0.185	0.073	1			
T_3	−0.264	−0.145	0.183	−0.112	0.14	0.164	0.554	1		
T_4	0.412	0.687*	0.702*	0.784**	−0.499	0.814**	0.2	0.293	1	
GH	−0.236	−0.023	0.316	−0.057	0.09	0.261	0.505	0.848**	0.326	1

**Shows significant correlation at $p < 0.05$, **shows highly significant correlation at $p < 0.01$.*
THI, temperature humidity index; BST, body surface temperature; RT, rectal temperature; RR, respiratory rate; ACTH, adrenocorticotropic hormone; T3, triiodothyronine; T_4, Thyroxine; GH, growth hormone.

Serum Antioxidant Indices of the Non-lactating Buffaloes in Different Seasons

Concentrations of antioxidant enzymes and heat shock proteins are one of the most important stress markers reflecting animals' comfort and climatic adaptability (24). We observed a significant increase in serum TAC in the non-lactating buffaloes in summer, indicating their ultimate response of antioxidant enzyme system to oxidative stress to remove excessive ROS. However, activities of CAT, SOD, and GPx were decreased significantly in summer, owing to the inability of antioxidant defense system to combat excessive ROS load (8). That's why such serum antioxidant enzymes have been used as physiological biomarkers to reveal the extent of oxidative stress in animals (9). Similarly, we observed significantly high concentrations of MDA in summer and autumn, revealing a higher degree of oxidative stress as it is a major end product of lipid peroxidation in cells to damage cell membranes. Similar findings regarding high MDA levels in summer have been reported earlier in Murrah buffaloes (25). In our study, a combination of oxidant (MDA) and antioxidant (TAC, GHS-Px, SOD, and CAT) enzymes revealed the high level of oxidative stress in non-lactating buffaloes during summer. These data indicate the need to exploit some cooling practices more effective than mist spraying to completely eliminate adverse effects of heat stress in buffaloes, especially during summer when THI value exceeds 80.

TABLE 6 | Correlation of THI with serum antioxidant parameters of non-lactating buffaloes.

Items	THI	TAC	MDA	GPx	SOD	CAT
THI	1					
TAC	0.640*	1				
MDA	0.54	0.522	1			
GSH	−0.837**	−0.608*	−0.736**	1		
SOD	−0.729**	−0.709**	−0.554	0.890**	1	
CAT	−0.596*	−0.353	−0.877**	0.871**	0.677*	1

**Indicate significant correlation at $p < 0.05$, **indicate highly significant correlation at $p < 0.01$.*
TAC, total antioxidant capacity; MDA, malondialdehyde, SOD, superoxide dismutase, GPx, glutathione peroxidase; CAT, catalase.

Effect of THI on Hormonal and Biochemical Parameters of the Non-lactating Buffaloes

Exposure of an animal to high ambient temperature adversely affects the activity of its thyroid gland owing its great sensitivity to body temperature and metabolic heat increment (26). In our study, GH concentrations were decreased significantly in summer and autumn, revealing the most obvious sign of heat stress in the non-lactating buffaloes. Reduced plasma GH in summer was in agreement with earlier studies on buffaloes (27, 28). Plasma T_3 levels were also decreased in summer and autumn which is in agreement with earlier findings regarding the depression of T_3 in buffaloes during the summer stress period (29). Decreases in plasma T_3 and T_4 during heat stress were considered as an adaptive strategy to reduce basal metabolic rate and then net body heat production (30, 31). Contrary to our findings, no difference in plasma T_3 and T_4 in Nili-Ravi buffaloes was observed between hot humid and hot dry seasons (32). Fluctuations in T_3 and T_4 concentrations in response to heat stress are not abrupt as it takes time to change them and subsequently it requires several days to stabilize them at a new steady state (6).

In contrast to T_3, plasma T_4 was significantly higher in summer and autumn than in spring and winter. Similar findings have been reported earlier as a significantly higher level of T_4 was observed in Khuzestan buffalo bulls during the summer season as compared to winter (33). Moreover, it was reported that the concentration of T_3, TSH, and free T_4 index did not differ between summer and winter seasons in same study. Additionally, no difference between T_3 and T_4 levels and their ratio has been observed in different seasons (summer, monsoon, and winter) in Murrah buffalo bulls (34). Similarly, no difference in T_3 uptake (%) was observed in the summer and winter season in Friesian calves (35). But heat stress significantly reduced the T_4 level in summer as compared to winter season while T_3 uptake (%) showed no difference between summer and winter seasons as reported in buffaloes and Friesian cows (36). But in the same study, animals that were treated with water spray and a diaphoretic showed a 32.4% increase in T_4 levels (42% in free T_4 index) making its concentration significantly higher than observed in the summer season (35). Interestingly, T_3 concentrations (% T_3 uptake) showed a comparatively lower response to this treatment as only a 11% increase in its concentration was observed. Many other studies have also reported that use of evaporative cooling strategies (such as mist spraying or water sprinkling over animal) can significantly alleviate the adverse effects of heat stress and mitigate the decline in T_4 levels as observed in crossbred cows (37) and buffaloes (38). Keeping in view of above findings the possible reason for higher plasma T_4 levels observed in the present study during summer and autumn is the use of mist spraying over buffaloes whenever the ambient temperature exceeds 28°C. The same trend was not observed for T_3 levels because T_3 is generally seen as the most metabolically active thyroid hormone and it has a shorter half-life in the blood than T_4 (39). The provision of mist spraying or water sprinkling showed no effect on plasma T_3 levels in summer season (40). Additionally, wallowing has also shown to decrease free T_3 in swamp buffaloes (41). That's why we observed lower plasma T_3 in the summer and autumn while higher T_4 in Nili-Ravi buffaloes in the present study.

The liver enzymes ALT and AST are the most reliable markers of liver damage or necrosis. The ALT primarily exists in the liver, but AST exists in various tissues like heart, liver, kidney, and so on. In the present study, we observed similar ALT levels during spring, summer, and winter except for autumn during which higher ALT level was observed. However, a higher AST level was found in spring with no change in other seasons. These findings revealed no adverse effect of heat stress on liver health in buffaloes in the present study as values of ALT and AST were within normal physiological limits (42). Animal exposure to ambient temperature up to 30°C, have not shown any significant change in ALT and AST levels in crossbred cattle (43). These observations are in line with our findings as we observed no rise in levels of both liver enzymes during the summer season in buffaloes as the average ambient temperature did not exceed above 30°C in the present study. No changes in the concentration of AST have been observed in the Surti buffalo during summer and winter seasons. However, a significant increase in ALT content was observed in the summer (under hot dry and hot humid conditions when THI increased from 68 to 81) as compared to winter season (44). Similar findings regarding the increase in ALT in the summer season (hot periods of the year) have been reported in cattle (45, 46). Heat stress during metabolically demanding conditions including lactation, pregnancy, or growth can put adverse effects on liver physiology, which is not the case with dry buffaloes as no changes in liver enzymes were observed during the summer season in the present study. Both AST and gamma-glutamyl transferase (GGT) have shown excellent predictive value for liver damage in cattle but in case of buffalo GGT is reported to be a more reliable indicator of liver damage (47). As we did not measure GGT in this study which is a limitation of the present study and should be considered in future studies.

Significantly high plasma cortisol was observed in summer, suggesting a high level of oxidative stress. Stressful conditions like heat stress result in activation of hypothalamic–pituitary–adrenal axis (HPA) which results in increased secretion of hypothalamic peptides including corticotrophin releasing hormone (CRH) and

arginine vasopressin (AVP) into pituitary portal circulation, where they stimulate ACTH secretion. This, in turn, triggers an increase in cortisol release from the adrenal gland to act on multiple targets to restore homeostasis (48). Increased plasma cortisol levels observed in summer in the present study, indicated enhanced activity of HPA to facilitate homeostasis and mitigate the adverse effects of heat stress. Similar findings regarding high cortisol contents in summer have been reported earlier in Egyptian buffaloes (1), Murrah buffaloes (17, 29), and Sahiwal cows (13). Moreover, elevated cortisol levels have been observed in crossbred buffaloes (Nili-Ravi × Murrah) as compared to purebred Mediterranean buffaloes (49).

Contrary to cortisol, we observed significantly higher level of ACTH in buffaloes during summer. Regulation of ACTH secretion within the HPA axis is a multifactorial process, with the hypothalamic peptides CRH and AVP being important physiological stimulators, and cortisol along with other adrenal glucocorticoids as a major inhibitory factor by feedback loops (50, 51). Inhibition of the ACTH response to CRH and AVP by cortisol depends on the length of exposure: fast (within seconds to minutes), intermediate (2–10 h) and slow (hours to days) feedback (50). Both fast and intermediate feedbacks inhibit ACTH release rather than synthesis, while the slow feedback appears to affect both ACTH release and synthesis. The higher plasma cortisol levels results in negative feedback to subsequently lower the plasma ACTH levels through mediating complex interaction with CRH and AVP (52). It has also been reported earlier that ACTH increases in response to the onset of stress and then decreases in spite of continuing heat stimulus, indicating a negative glucocorticoid feedback and a decrease in the glucocorticoid-binding transcortin (53). This is the possible reason for lower ACTH levels observed in buffaloes during summer when plasma cortisol was quite higher in the present study.

We observed significantly low plasma insulin level in summer, in agreement with an early report (54). However, contrary findings like increase (23) or no change in insulin concentration (55) have been reported in cows under heat stress. Such variations are mainly attributed to different metabolic status, nutrition, physiological stage and thermo-tolerance ability of the animals.

Association of THI With Physiological and Biochemical Parameters

THI has been efficiently used to evaluate the level of heat stress in animals (56, 57). Our study showed positive associations of THI with physiological parameters (BST, RR, and RT) and serum TAC content. However, moderately negative correlations of THI with ACTH and cortisol were observed, a bit strange as early study has reported a positive association of THI with cortisol content (29). However, a negative correlation of THI with serum antioxidant enzymes was in agreement with earlier studies on dairy cows (57, 58) and buffaloes (29). The decrease in activity of antioxidant enzymes during summer revealed a reduced total antioxidant capacity to manage the excessive load of ROS which has been associated with reproductive acyclicity in buffaloes, owing to severe damage to follicular cells (59). Usually non-lactating buffaloes are neglected in terms of management of heat stress as compared to lactating animals, which subsequently affects their reproductive performance. In our study, we observed average THI above 80 in summer which was well-correlated with the physiological and oxidative responses of non-lactating buffaloes. Therefore, it is recommended that in summer, buffaloes should be allowed to swimming for some period, especially during the hot period of the day, to alleviate adverse effects of heat stress. Earlier studies have also suggested the use of cooling strategies during months from April to September to alleviate heat stress in dairy animals (60). To best of our knowledge, present study is the first report on the seasonal dynamics of physiological and metabolic responses of non-lactating Nili-Ravi buffaloes to oxidative stress under the tropical climate in South China. It provides practical insights on the adaptive physiology of buffaloes and has several implications regarding the alleviation of heat stress in buffaloes to enhance the efficiency of performance and avoid animal welfare issues under tropical climate. However, a relatively small sample size is a limitation of this study that needs to be accounted for in future studies.

CONCLUSIONS

High THI in summer was associated with significant increases in physiological parameters, serum MDA and cortisol concentrations in non-lactating buffaloes. Moreover, THI showed a highly negative association with serum total antioxidant capacity as it significantly reduced serum contents of GSH, SOD, and CAT in summer. Plasma T_3 and GH concentrations were also reduced in summer. Our study reveals that summer months (from June to September) are critical in buffalo management as THI value exceeds 80, so cooling practices are required to alleviate heat stress to avoid performance losses and animal welfare issues. However, further studies are required on a larger cohort of samples to corroborate the association of THI thresholds with physiological and biochemical parameters in the non-lactating buffaloes under tropical climatic conditions.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of the Guangxi Buffalo Research Institute, Chinese Academy of Agriculture Sciences China (Approval No. BRI2017006). All animals studied were the property of the Guangxi Buffalo Research Institute.

AUTHOR CONTRIBUTIONS

ML, ZT, and CY: conceptualization. FH: data curation. ML: formal analysis. CY: funding acquisition. ML, ZT, FX, and LP: investigation. ML, YG, FX, and LP: methodology. XL: project administration. YG, ZT, XL, FX, LP, and CY: resources. FH: software and writing—original draft. CY: supervision and validation. FH and XL: visualization. ML and CY: writing—review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from the National Key Research and Development Program of China (2016YFD0500507 and 2018YFD0501602). The APC will be covered by the same grant.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 25 September 2020
doi: 10.3389/fvets.2020.539496

Knockdown of CYP19A1 in Buffalo Follicular Granulosa Cells Results in Increased Progesterone Secretion and Promotes Cell Proliferation

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Cytochrome P450 aromatase 19A1 (CYP19A1) is a critical enzyme in estrogen synthesis. However, the effect of CYP19A1 on cell growth and hormone secretion of buffalo follicular granulosa cells (BFGCs) is poorly understood. The objective of this study was to assess the role of CYP19A1 in cell proliferation and hormone secretion of BFGCs by knocking down CYP19A1 mRNA expression. The mRNA expression level of CYP19A1 gene was knocked down in BFGCs using the siCYP19A1-296 fragment with the best interference efficiency of 72.63%, as affirmed by real-time quantitative PCR (qPCR) and cell morphology analysis. The CYP19A1 knockdown promoted the proliferation of BFGCs through upregulating the mRNA expression levels of six proliferation-related genes (CCND1, CCNE1, CCNB1, CDK2, CDKN1A, and CDKN1B). Moreover, CYP19A1 knockdown increased ($P < 0.05$) the concentrations of progesterone secretion (P4) in BFGCs through increasing the mRNA expression levels of three steroidogenic genes (HSD17B1, HSD17B7, and CYP17A1). Our data further found that the FSH could inhibit the mRNA expression level of CYP19A1 in BFGCs, while LH obtains the opposite effect. These findings showed that the CYP19A1 knockdown had a regulatory role in the hormone secretion and cell proliferation in BFGCs.

Keywords: buffalo, CYP19A1, follicular granulosa cell, progesterone secretion, cell proliferation

OPEN ACCESS

Edited by:
Antonio Minervino, Federal University of Western Pará, Brazil

Reviewed by:
Zhao Zhihui, Guangdong Ocean University, China
Rinaldo Batista Viana, Federal Rural University of the Amazon, Brazil

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *01 March 2020*
Accepted: *24 August 2020*
Published: *25 September 2020*

Citation:
Lu X, Duan A, Ma X, Liang S and Deng T (2020) Knockdown of CYP19A1 in Buffalo Follicular Granulosa Cells Results in Increased Progesterone Secretion and Promotes Cell Proliferation. Front. Vet. Sci. 7:539496. doi: 10.3389/fvets.2020.539496

INTRODUCTION

With the action of reproductive hormones among the hypothalamic-pituitary-gonadal axis, follicular cells could promote the expression of steroid hormone synthase, guide the production of steroid hormones, and further regulate the growth and development of follicular. Estradiol (E2) produced by the interaction of steroid hormone synthase in follicular membrane cells and granulosa cells, which help in promoting the proliferation of granulosa cells and regulating the differentiation of follicular cells in the ovaries (1). In addition, E2 also promotes the sexual performance of female animals and physiological changes in the reproductive tract, the establishment of pregnancy, the development of breast catheters, calcium absorption (2).

Cholesterol side-chain cleavage enzyme is commonly referred to as P450SCC, which is a member of the cytochrome P450 family enzyme. The cytochrome P450 family 11 subfamily A member 1 (CYP11A1) converts cholesterol from follicular theca cells into progesterone (P5), which is the first and rate-limiting enzymatic step in the production of all steroid hormones (3). Subsequently, P5

is converted into progesterone (P4) by the action of 3beta-hydroxysteroid (HSD3B1) in follicular membrane cells, which is further transformed into androstenedione (A4) under the action of cytochrome P450 17A1 (CYP17A1) in granulosa cells (4). Notably, A4 is a precursor of estrone and estradiol that play a critical role in the estrogen synthesis (5, 6). A previous study has demonstrated that the Cytochrome P450 19A1 (CYP19A1, known as estrogen synthase) is a key enzyme that can convert A4 to Estradiol 1 (E1) during the estrogen synthesis (7). E1 could be converted into E2, by active of 17b-hydroxylase steroid dehydrogenase family, especially by the action of HSD17B1 (4). Alternatively, CYP19A1 may convert A4 to testosterone (8) that can further be transformed into E2 (9). Notably, numerous studies have reported that the E2 content tended to increase by increasing the mRNA expression levels of CYP19A1 and HSD17B1 in the bovine follicles grow from small to large initial stages (10–12). These results suggested that both CYP19A1 and HSD17B1 were the key enzymes in E2 production. However, limited information on the effects of HSD17B1 and CYP19A1 on the E2 secretion has been reported. Although the buffalo CYP19A1 has recently been successfully cloned and its tissue distribution analysis has been performed (13), the regulation function of CYP19A1 and its effect on the E2 secretion haven't been declared. Therefore, this study aims to investigate the effect of CYP19A1 on cell proliferation and hormone secretion in buffalo granulosa cells using RNA interference (RNAi) technology.

MATERIALS AND METHODS

Cell Culture

A total of 24 ovaries from 12 Chinese local buffaloes were collected from a slaughterhouse in Guangxi Nanning, immediately stored in sterile 0.9%(w/v) NaCl at 37°C and transported to the laboratory within 4 h. The BFGCs were cultured and purified according to previously reported methods (14). Purified BFGCs were cultured in DMEM media supplemented with 10% FBS, penicillin (100 $U \cdot mL^{-1}$) and streptomycin (100 $\mu g \cdot mL^{-1}$) at 38.5°C in humidity and an atmosphere of 5% CO_2 with a humidified incubator. Cells were used for subsequent experiments when the cell congruence reaches 90%. The remaining cells were stored immediately in liquid nitrogen.

siRNA Experiments

A total of 3 siRNA fragments for buffalo CYP19A1 (GenBank accession number: KC020137.1) and control siRNA (NC) were recruited from the GenePharma Company (Shanghai, China). The sequences of siRNA fragments were listed in **Supplementary Table 2**. BFGCs were transfected with three CYP19A1 siRNA and NC at 50 nM concentration, using the Lipofectamine RNAiMAX Transfection Reagent (ThermoFisher, MA, USA) following the manufacturers' instructions. Total RNA were obtained from siRNA-transfected BFGCs after 72 h treatments. The siRNA transfection efficiency was monitored by qRT-PCR.

RNA Isolation

Total RNA was isolated using the PureLink RNA Mini kit (Ambion, USA) following the manufacturer's instruction. RNA quantity and quality were evaluated using the NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Reverse Transcription and Quantitative Real-Time PCR

A total of 100 ng RNA for each sample was used for the preparation of cDNA by using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, MA, USA). The qRT-PCR was conducted using the PowerUp SYBR Green Master Mix with the 20 μL reaction volume: PowerUp SYBR Green Master Mix 10 μL, RNase-free water 8 μL, upstream and downstream primers 0.5 μL, and cDNA 1 μL. The reaction procedure was run on the LightCycler 480 instrument (Roche, CH) under the following conditions: 95°C 30 s, 40 cycles of 95°C 5 s, 60°C 30 s. GAPDH was utilized for the reference gene, and the relative expression levels of the CYP19A1 gene in each experimental group were calculated by the $2^{-\Delta\Delta Ct}$ method (15). Primer pairs for all detected genes were shown in **Supplementary Table 1**.

Cell Morphology Analysis

Changes in cell morphology of each experimental group were observed by microscopy after 72 h transfection, and the effects of each interference group on the growth morphology of BFGCs were compared.

Cell Proliferation Assay

BFGCs were plated in 96-well culture plates and incubated in a growth medium complemented with 10% FBS until 60% confluence. Cell proliferation was evaluated after 72 h transfection, using the CCK-8 cell proliferation Kit (KeyGEN BioTECH, Jiangsu, China) according to the manufacturer's instruction. Add cell proliferation detection fluid with 10 μL per cell hole, incubated for 4 h at 37°C. Overall, the proliferation for each optimal interference group was detected at the 24, 48, and 72 h, respectively. The cell activity is expressed by optical density (OD) value in 450 nm wavelengths.

Cell Apoptosis Assay

Annexin V-EGFP Kit (KeyGEN BioTECH, Jiangsu, China) was selected for the cell apoptosis analysis according to the manufacturer's instruction. Briefly, the cells were firstly treated with the pancreatic enzymes without EDTA after the CYP19A1 knockdown for 72 h, followed by washing two times with PBS, suspending cells with 500 μL binding Buffer, blending the cells with the 5 μL annexin V-EGFP Mix and 5 μL propidium iodide. After the mixed reaction was in room temperature and light avoidance of 10–20 min, BFGCs were observed and detected under the fluorescence microscope. For them, green light cells indicate early apoptosis cells, and red-light cells indicate apoptosis in the mid and late-stage and necrotic cells. Effects of CYP19A1 knockdown on apoptosis in experimental groups were analyzed and compared, and the calculation formula was as follows: the rate of apoptosis cells = number of apoptosis

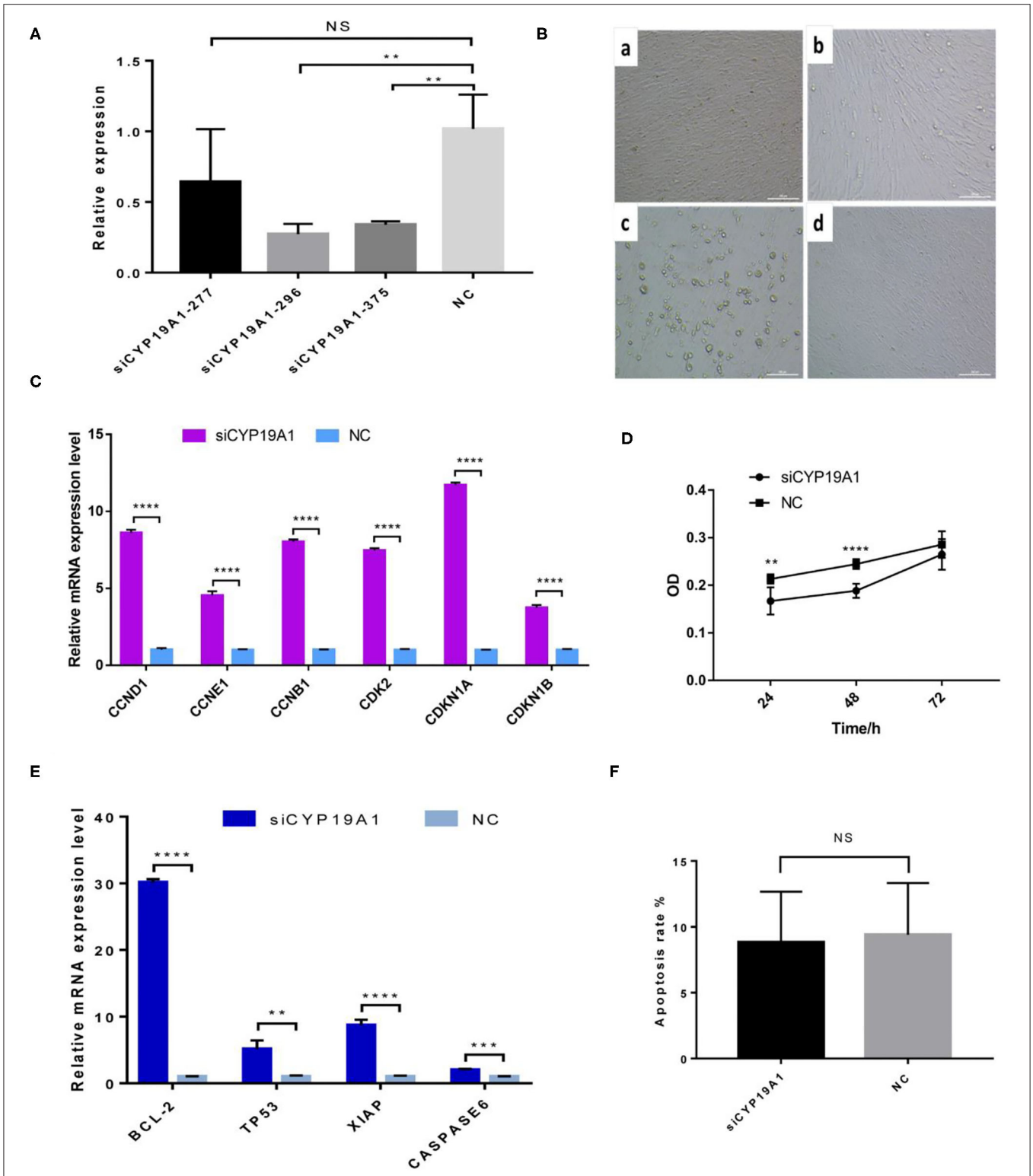

FIGURE 1 | CYP19A1 induced cell proliferation and apoptosis of BFGCs. **(A)** A screen of CYP19A1 siRNA fragments in BFGCs. **(B)** Changes of cell morphology after CYP19A1 interference for 72 h. 200 × (bar = 100 μm). Note a: BFGCs transfected with siCYP19A1-277 for 72 h; b: BFGCs transfected with siCYP19A1-296 for 72 h; c: BFGCs transfected with siCYP19A1-375 for 72 h; d: BFGCs transfected with NC for 72 h. **(C)** Effect of CYP19A1 gene interference on proliferation-related genes expression. **(D)** Effect of siCYP19A1 on BFGCs by CCK-8 cck8 detection. **(E)** Effect of CYP19A1 gene interferen ce on apoptosis-related genes expression. **(F)** Comparison of apoptosis rates between test group and native control group. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; NS, no significant.

cells/numbers of total cells, the rate of living cell = number of live cells/numbers of total cells.

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of E2 and P4 were determined in the cell culture supernatants using ELISA kit (Beijing Kangpu Tongchuang Biotechnology Co., Ltd, Beijing, China) after 72 h transfection. In this study, three repeating experiments were designed, each of which was repeated three times. The results were calculated based on standard density: 120, 80, 40, 20, 10 ng/L.

Statistical Analysis

The data were expressed as mean ± SEM. Tukey *post hoc* test and one-way ANONA analysis were used to determine the statistical significance. Each test was conducted in triplicate. Data analysis was performed by SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 7.0 (GraphPad Prism Software Inc., San Diego, USA).

RESULTS

siRNA Interference Efficiency Detection of CYP19A1

The interference efficiency of three siRNA fragments of CYP19A1 was verified by BFGCs transfection. As shown in **Figure 1A** and **Supplementary Table 3**, all the siRNA fragments had a inhibitory effect on the CYP19A1 of BFGC, and siCYP19A1-296 showed the best interference efficiency (72.63%, $P = 0.007$) compared to that of siCYP19A1-277 (35.86%, $P = 0.217$) and siCYP19A1-375 (66.03%, $P = 0.0086$). Moreover, the BFGCs treated by siCYP19A1-296 remained a flat elongated shape after 72 h, with a small number of cells began to enter the early apoptosis (**Figure 1B**). These results indicated that the siCYP19A1-296 can serve as the best siRNA fragments and used for further analysis.

CYP19A1 Knockdown Altered BFGCs Proliferation

The effect of CYP19A1 knockdown on cell proliferation was investigated at three time-points (24, 48, and 72 h). Compared with the NC group, BFGCs treated by the CYP19A1 knockdown increased cell population ($P < 0.05$) in the culture medium (**Figure 1D** and **Supplementary Table 4**). Subsequently, mRNA levels of six proliferation-related genes (CCND1, CCNE1, CCNB1, CDK2, CDKN1A, and CDKN1B) were further determined after CYP19A1 knockdown using qPCR. The results indicated that the increases ($P < 0.05$) in the mRNA expression level of the studied genes were observed after CYP19A1 silencing (**Figure 1C**). These findings indicate that CYP19A1 plays a key role in BFGC growth.

Effects of CYP19A1 Knockdown on BFGC Apoptosis

To determine apoptosis regulation of CYP19A1 in BFGC, apoptosis cells were detected by Annexin V-EGFP Kit. **Figure 1E** showed no effect on the apoptosis rate ($P > 0.05$) between them (**Figure 1F**). Interestingly, when the mRNA expression levels of apoptosis inducers were quantified, we found that the CYP19A1 knockdown increased ($P < 0.05$) the expression of the apoptosis genes (TP53 and CASPASE6) and anti-apoptosis genes (Bcl-2 and XIAP), and the latter had higher ($P < 0.05$) expression level compared to that of the former.

CYP19A1 Knockdown Increased the Concentrations of Progesterone in BFGC

To verify the effect of silencing CYP19A1 on steroid hormone levels, the concentrations of E2 and P4 in the culture medium at 48 h post-transfection were observed. Results showed that P4 levels in the CYP19A1 knockdown group were higher ($P < 0.05$) than that of the NC group after transfection. There was no difference between the E2 group and NC (**Figure 2A**; **Table 1**). The mRNA expression of steroidogenic

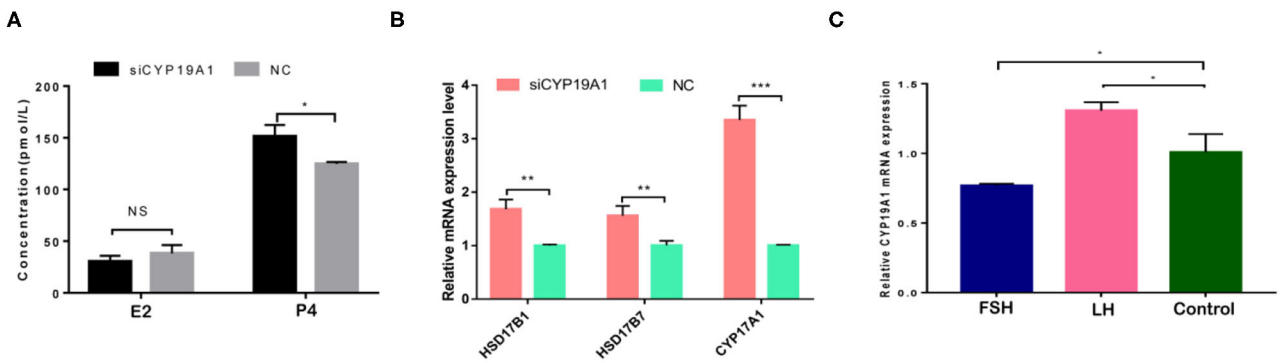

FIGURE 2 | CYP19A1 regulated hormone secretion of BFGCs. **(A)** Effects of E2 and P4 secretion from BFGCs with CYP19A1 interference; **(B)** Effect of CYP19A1 knockdown on steroidogenic gene expression. **(C)** Effects of adding LH and FSH on CYP19A1 expression. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

genes, including HSD17B1, HSD17B7, and CYP17A1 was further analyzed. The results showed that CYP19A1 knockdown increased ($P < 0.05$) in HSD17B1, HSD17B7, and CYP17A1 at the mRNA expression level (**Figure 2B**). Moreover, we also determined the mRNA expression of several upstream genes (FSH and LH). The results showed that the FSH decreased ($P < 0.05$) the expression of the CYP19A1. Whereas, the LH increased ($P < 0.05$) the CYP19A1 gene expression (**Figure 2C**). These results confirmed that P4 secretion was controlled by CYP19A1 in BFGCs.

TABLE 1 | Effects of E2 and P4 secretion from BFGCs with CYP19A1 interference.

Groups ($n = 3$)	Concentration of E2 (pmol/L) (Mean ± SD)	Concentration of P4 (pmol/L) (Mean ± SD)
siCYP19A1	30.35 ± 3.20	151.46 ± 6.32[a]
NC	38.22 ± 4.63	124.81 ± 1.06[b]

[a,b]*Values within each column are significantly different ($p < 0.05$).*

DISCUSSION

Evidence indicated that the growth and development of follicles are mediated by hormones and growth factors secreted from granulosa cells (16), including E2 and P4 (17, 18). Previous studies have demonstrated that both E2 and P4 play a vital role in the steroid hormone synthesis involved in the regulation of granulosa cells proliferation and differentiation in the ovaries (1, 19, 20). CYP19A1 is a critical enzyme in the E2 synthesis, however, there are limited reports about the molecular mechanism of CYP19A1 regulating BFGCs proliferation, apoptosis, and hormone synthesis. To explore these issues, we designed and synthesized the interference fragment, siCYP19A1, to silence CYP19A1 mRNA expression in BFGCs. Then, we investigated the effects of CYP19A1 knockdown on cell proliferation, apoptosis, and hormone production of BFGCs. In the present study, we found that there was a strong inhibitory effect on the proliferation of BFGCs after CYP19A1 knockdown for 24 and 48 h, and this inhibition would decrease as the cells continued to divide after 72 h. This finding suggested that the CYP19A1 gene could be considered as candidate genes affecting the BFGC growth.

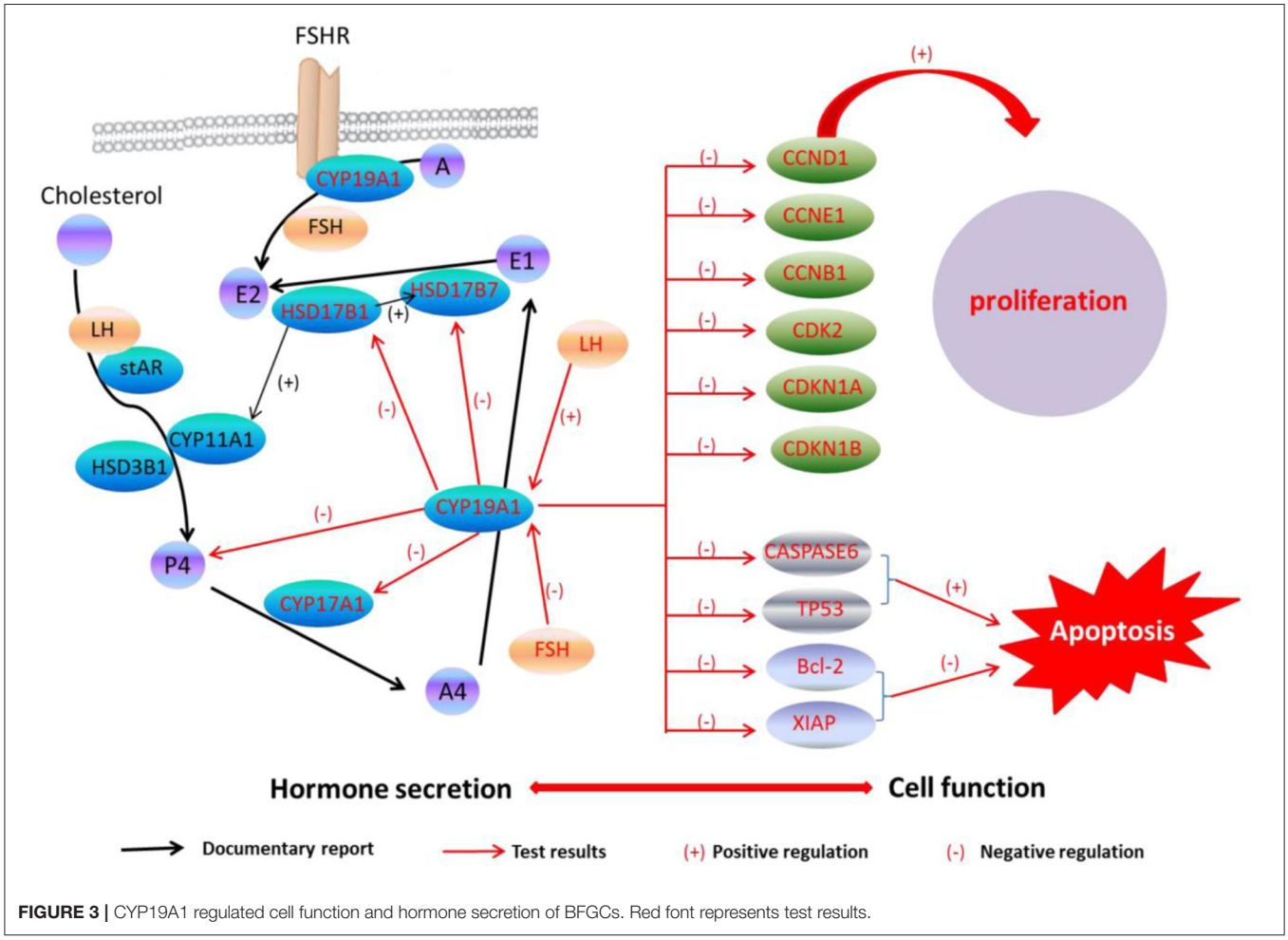

FIGURE 3 | CYP19A1 regulated cell function and hormone secretion of BFGCs. Red font represents test results.

To gain insight into the mechanisms that CYP19A1 prevents the BFGC growth, we assessed the changes in mRNA expression of key proliferation-related genes (CCND1, CCNE1, CCNB1, CDK2, CDKN1A, and CDKN1B). Our data showed all the proliferation-related genes appeared to be up-regulated after the CYP19A1 knockdown. Previous studies have demonstrated that all of them play important regulatory roles in cell proliferation and the cell cycle (21–24). For example (25), found that neuron growth factors (NGF) promote the proliferation of granulosa cells (GC) by down-regulating ESR2 and CDKN1A. A similar result was also supported by our data. These results showed that CYP19A1 was an essential gene to maintain the growth and proliferation of BFGCs.

Apoptosis is a cellular mechanism of ovarian follicular atresia and a physiological form of cell death. Previous studies have demonstrated that Bcl-2 gene family members regulate the apoptosis of granulosa cells (26, 27). As the CYP19A1 knockdown has been shown to impact on the cell proliferation, we hypothesized that the knockdown of CYP19A1 may promote cell survival by reducing apoptosis rate of BFGCs. However, we determined that the knockdown of CYP19A1 had no effect on the apoptosis of BFGCs. At the mRNA level, we observed the mRNA expression level of anti-apoptosis genes (Bcl-2 and XIAP) was higher ($P < 0.05$) than apoptosis genes (TP53 and CASPASE6) after CYP19A1 knockdown. Taken together, we speculated that the knockdown of CYP19A1 may affect the mRNA expression level of various apoptosis-related factors, but there is no effect on apoptosis rates in BFGC.

Granulosa cells are key cells in hormone secretion, and CYP19A1 plays an important role in hormone circulation in the body (4, 28). In this study, steroid hormone secretion detection analysis revealed that the CYP19A1 knockdown increased the secretion of P4 without changing the sec retion of E2 in BFGCs. Meanwhile, our data showed an increase ($P<0.05$) in expression of steroid-related gene HSD17B1, HSD17B7, and CYP17A1after CYP19A1 knockdown, accompanied by an increase in P4 secretion. However, the reasons for CYP19A1 knockdown not causing the change of E2 secretion have yet to be further verified, maybe HSD17B1 and HSD17B7 were antagonistic in regulating E1 to E2 conversions, there was a regulatory link that has not been confirmed.

A previous study has found that the FSH adding may raise the E2 and P4 levels during the granulosa cells culture *in vitro*, while the secreting level of E2 and P4 was increased by adding the LH in a small quantity (29). Because of CYP19A1 is a key enzyme which involves in E2 synthesis, the expression of CYP19A1 was regulated by FSH and LH in the mRNA level. Our data further showed that FSH adding to the medium reduced the relative expression of CYP19A1 in BFGCs, whereas adding LH could increase the relative expression of CYP19A1 (**Figure 3**). It can be inferred that CYP19A1 plays an important role in maintaining the stability of hormone secretion in the body.

CONCLUSION

In this work, we confirmed that the CYP19A1 knockdown may promote BFGCs cell proliferation through regulating the proliferation-related gene expressions. We also confirmed that the CYP19A1 knockdown may increase P4 synthesis by controlling the expression of steroidogenic genes. Our findings provided insight to reveal the relationship between the structural function of granulosa cells and hormone secretion.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

This study has been approved by the Ethics Committee of Buffalo Research Institute, Chinese Academy of Agricultural Sciences (BRI-CAAS), Nanning, China (approval code GXBRI-200701).

AUTHOR CONTRIBUTIONS

TD and XL were in charge of designing experiments and analyzing data. XL and AD were responsible for preparing the initial draft of the manuscript. XL and TD were in charge of approving the final draft of the manuscript and were responsible for cell culture, cell proliferation, apoptosis assays, and data collection. XM and SL were responsible for qPCR analysis and enzyme assay testing and data collection. All the authors have examined the manuscript.

FUNDING

This research was supported by the Natural Science Foundation of Guangxi, China (2017GXNSFBA198191 and 2017GXNSFBA198022).

ACKNOWLEDGMENTS

We thank the people who provided us the buffalo ovary samples. We would like to thank the Natural Science Foundation of Guangxi, China, and the central guidance on implementing local fund projects, China for funding this research. Our thanks also go to the Key Laboratory of Buffalo Genetics, Breeding and Reproduction Technology, Ministry of Agriculture in Nanning, China for the instrument support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.539496/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 15 October 2020
doi: 10.3389/fvets.2020.547047

Lipid Accumulation and Injury in Primary Calf Hepatocytes Challenged With Different Long-Chain Fatty Acids

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Fatty liver disease is one of the most common disorders afflicting dairy cows during the postpartum period, and is associated with increased blood non-esterified fatty acid (NEFA) uptake by the liver. Major long-chain fatty acids (LCFA) in NEFA are palmitic (PA), palmitoleic (POA), stearic (SA), oleic (OA), and linoleic (LA) acid. In order to investigate the characteristics of lipid accumulation and injury caused by these NEFA, primary calf hepatocytes were isolated and challenged for 12 h with 1.2 mmol/L PA, POA, SA, OA, LA, or a mixture of these LCFA (NEFA). Compared with POA, OA, and LA, culture with PA and SA led to greater abundance of CCAAT-enhancer binding protein, glucose-regulated protein 78 mRNA, and stearoyl-CoA desaturase 1 mRNA along with greater concentrations of H_2O_2, malondialdehyde and reactive oxygen species (ROS). Although culture with POA, OA, and LA led to lower very low density lipoprotein (VLDL) concentration in cell culture medium, POA and OA led to greater concentrations of triacylglycerol, protein abundance of sterol regulatory element-binding protein 1c, fatty acid synthase, acetyl coenzyme A carboxylase 1, ApoB100, and sortilin 1 (SORT1). Compared with individual fatty acids, culture with NEFA led to an intermediate degree of lipid accumulation and hepatocytes damage. Overall, the data suggest that saturated fatty acids cause more severe oxidative and ER stress. However, unsaturated fatty acids cause serious lipid accumulation. Furthermore, a fatty acid balanced nutrient regulation was suggested useful improve liver health of transition period dairy cows.

Keywords: non-alcoholic fatty liver disease, fatty acids, lipid accumulation, endoplasmic reticulum stress, negative energy balance, primary calf hepatocytes

OPEN ACCESS

Edited by:
Domenico Vecchio, Istituto Zooprofilattico Sperimentale del Mezzogiorno, Italy

Reviewed by:
F. Capela e Silva, Universidade de Évora, Portugal
Miao Long, Shenyang Agricultural University, China

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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *30 March 2020*
Accepted: *14 September 2020*
Published: *15 October 2020*

Citation:
Zhang B, Yang W, Wang S, Liu R, Loor JJ, Dong Z, Zhao Y, Ma X, Xia C and Xu C (2020) Lipid Accumulation and Injury in Primary Calf Hepatocytes Challenged With Different Long-Chain Fatty Acids. Front. Vet. Sci. 7:547047. doi: 10.3389/fvets.2020.547047

INTRODUCTION

Negative energy balance (**NEB**) is a subhealthy state commonly experienced by transition period cows as a consequence of insufficient dry matter intake to sustain the high energy requirements for maintenance of body functions and milk production. With severe NEB, a large number of non-esterified fatty acid (**NEFA**) produced by fat mobilization, usually lead to the occurrence of fatty liver (1). Fatty liver is the most-important metabolic disease afflicting dairy cows early postpartum (2). It is usually characterized by increased plasma NEFA concentrations, accumulation of lipid

droplets in hepatocytes (3), and hepatomegaly and inflammation (steatohepatitis) (4). In rats, evidence indicates that oxidative stress and endoplasmic reticulum stress may promote steatosis, reactive oxygen species (**ROS**) production and development of non-alcoholic steatohepatitis (**NASH**) (5). Steatosis is an early stage of fatty liver, which leads to lipid peroxidation, inflammation, hepatic stellate cell activation, and fibrosis through intracellular NEFA accumulation (6). The high concentration of plasma NEFA in transition period cows contains a variety of fatty acids, with palmitic (**PA**), palmitoleic (**POA**), stearic (**SA**), oleic (**OA**), and linoleic (**LA**) acid being the most abundant (7–9). Clearly, these represent major fatty acid classes, saturated (**SFA**, PA, SA), monounsaturated (**MUFA**, POA, OA), and polyunsaturated (**PUFA**, LA). Some data from human studies indicate alterations in plasma long-chain fatty acid profiles among the healthy, non-alcoholic fatty liver and NASH (10), but it is unclear if the major fatty acids in the NEFA fraction of dairy cows have equal potency to induce hepatocytes lipid accumulation and injury.

Endoplasmic reticulum (**ER**) stress is associated with fatty liver, NASH and other inflammation-related diseases. It is a response to abnormal protein synthesis, folding, modification, or transport (11, 12). In liver *in vivo* and *in vitro* study of cow, ER stress can lead to fat accumulation leading to activation of the unfolded protein reaction (**UPR**) and alterations in lipid metabolism (4). Increased expression of ER chaperones such as glucose-regulated protein (**GRP78**) that cope with accumulation of unfolded or incorrectly folded proteins, is a marker of ER stress (13, 14). C/EBP homologous protein (**CHOP**) is the main signal protein that connects ER stress and hepatocyte injury (15). Endoplasmic reticulum stress can induce hepatic steatosis by reducing lipid outflow or catabolism (16). Present *in vivo* study confirms the occurrence of ER stress in dairy cows with severe fatty liver, *in vitro*, high fatty acids induced calf hepatocytes ER stress promoted lipid accumulation (4).

Sterol regulatory element-binding protein-1c (**SREBP-1c**) is a transcription factor that can regulate hepatic fatty acid synthesis. SREBP-1c target genes include acetyl coenzyme A carboxylase 1 (**ACC1**) and fatty acid synthase (**FAS**), which regulate liver fat accumulation and participate in the progression of hepatic steatosis and hypertriglyceridemia (17, 18). Very low density lipoprotein (**VLDL**) is one of the major lipoproteins that transport endogenous triacylglycerol (**TG**) out of the liver (19). Apolipoprotein B100 (**ApoB100**) is the major protein in VLDL and is essential for loading of TG into the growing VLDL molecule (20, 21). Sortilin (**SORT1**) is a receptor for ApoB100, and regulates low density lipoprotein-cholesterol (**LDL-C**) levels by mediating lipid particle synthesis and transport (22). In contrast, proprotein convertase subtilisin/kexin type 9 (**PCSK9**) can reduce uptake and metabolism of LDL-C by promoting lysosomal digestion of LDLR (23).

In transition period cows, PA, POA, SA, OA, and LA are the most abundant fatty acid in plasma, some of which have demonstrated more potency in terms of altering lipid metabolism *in vitro*. For instance, OA induced more pronounced alterations in lipid metabolism in human hepatocytic cell lines than a mixture of NEFA underscoring potential differences in liver metabolism among long-chain fatty acids (24). Obviously, each FA can differentially affect the metabolism of liver. Our hypothesis was that long-chain FA would have different effects on lipid injury of cow hepatocytes dependent on the specific FA. Therefore, the primary aim of this *in vitro* study was to investigate alterations in lipid metabolism, oxidative stress and ER stress in calf hepatocytes incubated with various fatty acids.

MATERIALS AND METHODS

Animal Care and Experimental Protocols

The study protocol was approved by the Ethics Committee for the Use and Care of Animals, Heilongjiang Bayi Agricultural University (Daqing, China). The animal studies were performed in accordance with the Guiding Principles of Animals adopted by the Chinese Association for Laboratory Animal Sciences.

The experimental animals were purchased from a farm in Heilongjiang Province (China). Newborn healthy female Holstein calves ($n = 4$, 1 d old, 40–50 kg, rectal temperature 38.7–39.7°C) were fasted for 24 h. After intramuscular injection of sodium thiopentobarbital (50 mg/kg) for anesthesia, they received an intravenous injection of 1,500 IU/kg heparin sodium for anticoagulation. The caudate lobes of the liver were obtained by surgical hepatectomy, and quickly moved to a super-clean bench. The animals were euthanized immediately by intravenous injection of saturated potassium chloride (150 mg/kg of

TABLE 1 | Sequences of primers used for real-time PCR amplification.

Gene	Forward (5′-3′)	Reverse (5′-3′)
β-actin	GCTAACAGTCCGCCTAG AAGCA	GTCATCACCATCGGC AATGAG
GRP78	GCATCGACCTGGGTACC ACCTA	CCCTTCAGGAGTGAAA GCCACA
SREBP-1c	GCAGCCCATTCATCAGCC AGACC	CGACACCACCAGCATCA ACCACG
FAS	ACAGCCTCTTCCTGTTT GACG	CTCTGCACGATCA GCTCGA
SORT1	TCCTTGGACCGACATCTC TACACC	TCGCATTCACTGTTCTCA GGCTTC
ACC1	TCCTGCTGCTATTGCT ACTCCA	CAGTCCCCGCACTCA CATAA
SCD1	CCGCCCTGAAATGAG AGATG	AGGGCTCCCAAGTGTA ACAGAC

Abbreviations: ACC1, acetyl coenzyme A carboxylase 1; ApoB100, Apolipoprotein B100; CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; FAS, fatty acid synthase; FBS, fetal calf serum; GRP78, glucose-regulated protein; LA, linoleic; LCFA, long-chain fatty acids; LDL-C, low density lipoprotein-cholesterol; MDA, malonaldehyde; MTP, microsomal triglyceride transfer protein; NASH, non-alcoholic steatohepatitis; NEFA, non-esterified fatty acid; OA, oleic; PA, palmitic; PBS, phosphate-buffered saline; PCSK9, proprotein convertase subtilisin/kexin type 9; POA, palmitoleic; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; SA, stearic; SREBP-1c, Sterol regulatory element-binding protein-1c; SORT1, Sortilin; TG, triacylglycerol; UPR, unfolded protein reaction; VLDL, lower very low density lipoprotein.

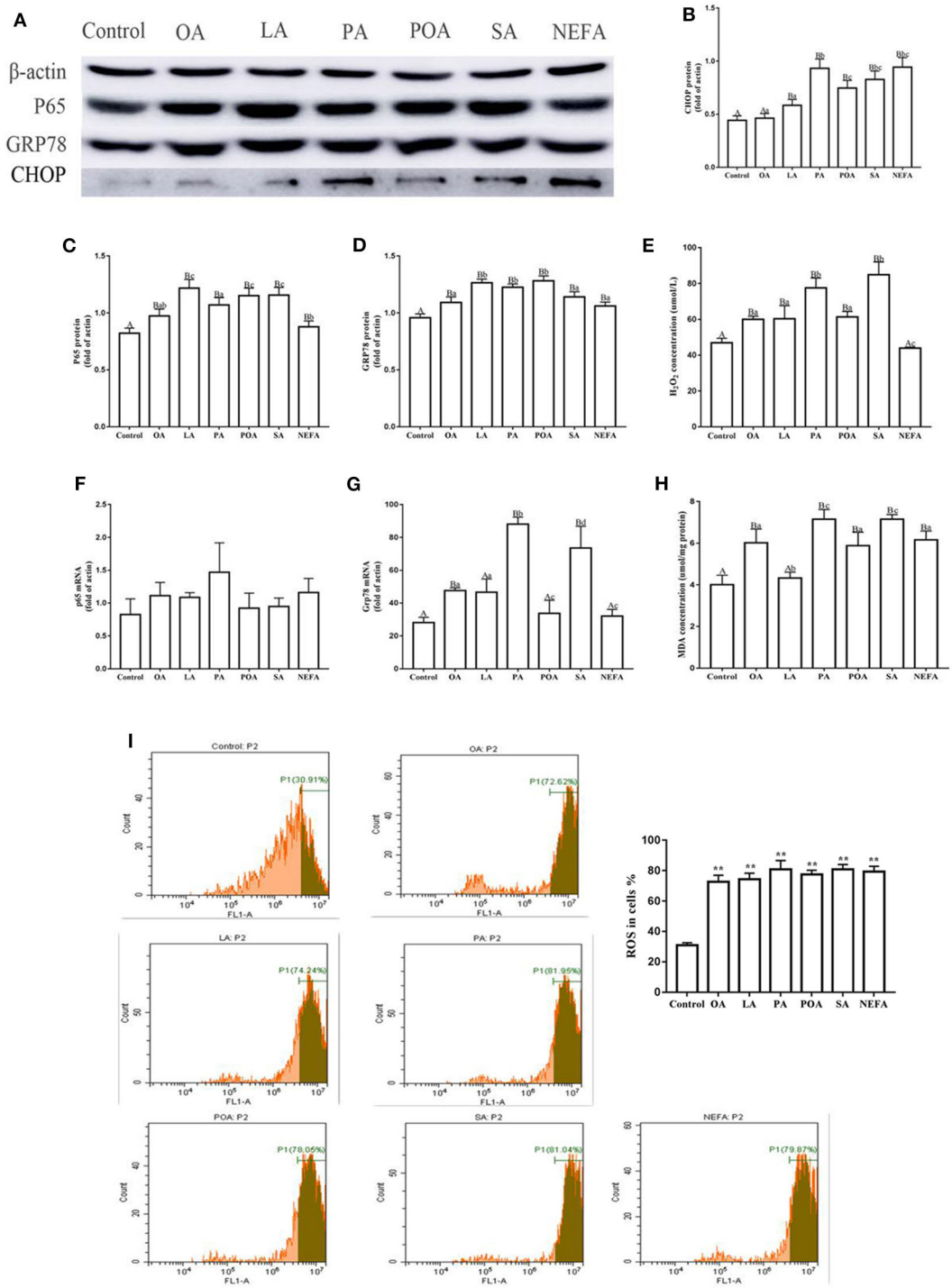

FIGURE 1 | Effects of different fatty acids on stress and inflammatory response of primary hepatocytes. Cells were treated with 1.2 m*M* fatty acids for 12 h ($n = 3$ ell preparations). **(A)** β-actin, P65, GRP78, and CHOP protein expression were assayed by western blotting. Gray-shaded bars represent the amount (%) of β-actin, P65, GRP78, and CHOP in primary hepatocytes. **(B–D)** CHOP, P65, and GRP78 protein expression in primary hepatocytes were assayed by western blotting and reported relative to β-actin. **(F,G)** P65 and GRP78 mRNA expression were assayed by real-time RT-qPCR. **(I)** ROS were assayed by fluorescence microscopy and flow cytometry. Fluorescence intensity is proportional to ROS activity in primary hepatocytes. $^{**}p < 0.01$ solvent and control group. **(E,H)** H_2O_2 (μmol/L) and MDA (μmol/mg protein) were assayed using commercial kits. Four assays were independently performed in each group. Uppercase letter vs. control $p \leq 0.05$, lowercase letter vs. fatty acid group $p \leq 0.05$, the same letter vs. no letter mark $p \geq 0.05$.

calf BW). Primary hepatocytes were isolated using a modified 3-step collagenase perfusion method as previously described (25). The perfusion solution A (140 m*M* NaCl, 6.7 m*M* KCl, 10 m*M* HEPES, 2.5 m*M* glucose, and 0.5 m*M* EDTA. pH 7.4; 37°C, 50 mL/min for 10–15 min) preheated at 37°C was used to rinse the blood surface of the caudate process surface, and the blood vessel of the cross section was leaked. The liver was then perfused with the same flow rate of 37° C pre-warmed perfusion solution B (140 m*M* NaCl, 6.7 m*M* KCl, 30 m*M* HEPES, 2.5 m*M* glucose, and 5 m*M* $CaCl_2$, pH 7.4; 37°C, 50 mL/ min for 5 min)until the liquid became clear. Then, the liver was perfused with perfusion solution C (0.1 g of collagenase IV dissolved in 0.5 L of perfusion solution B, pH 7.2–7.4; 20 mL/min for 15 min) to dissociate liver tissue structure until the liquid became muddy. The digestion was terminated with 4°C pre-cooled fetal calf serum (**FBS**; Hyclone Laboratories, Logan, UT), the liver capsule was torn off with scissors and forceps, the tissue was cut, and the blood vessels and connective tissue were removed. The shredded liver tissue was resuspended in 4°C pre-cooled RPMI1640 medium and filtered sequentially with 100 mesh (150 μm) and 200 mesh (75 μm) cell sieves.

The suspension of hepatocytes was washed with basic medium (Hyclone Laboratories), centrifuged, and resuspended in the adherent medium, counted and seeded into a 6-well tissue culture plate (2 mL per well) at 1×10^6 cells/mL using adherent medium (RPMI-1640 basic medium supplemented with 10% FBS, 1 μM of insulin, 1 μM of dexamethasone, 10 μg/mL of vitamin C) and incubated at 37°C in 5% CO_2 for 4 h. Then growth medium (RPMI-1640 basic medium supplemented with 10% FBS) was changed every 24 h until the primary hepatocytes were cultured for 44 h.

In order to explore the different effects of single fatty acids and NEFA, we screened five fatty acids as the experimental group according to the composition of NEFA, which included OA, LA, PA, POA and SA (Sigma-Aldrich, St. Louis, MO). Stock NEFA solution was prepared by diluting individual fatty acids in 0.1 M KOH at 60°C. pH of the solution was adjusted to 7.4 with hydrochloric acid (1 M). The stock NEFA (52.7 mM)

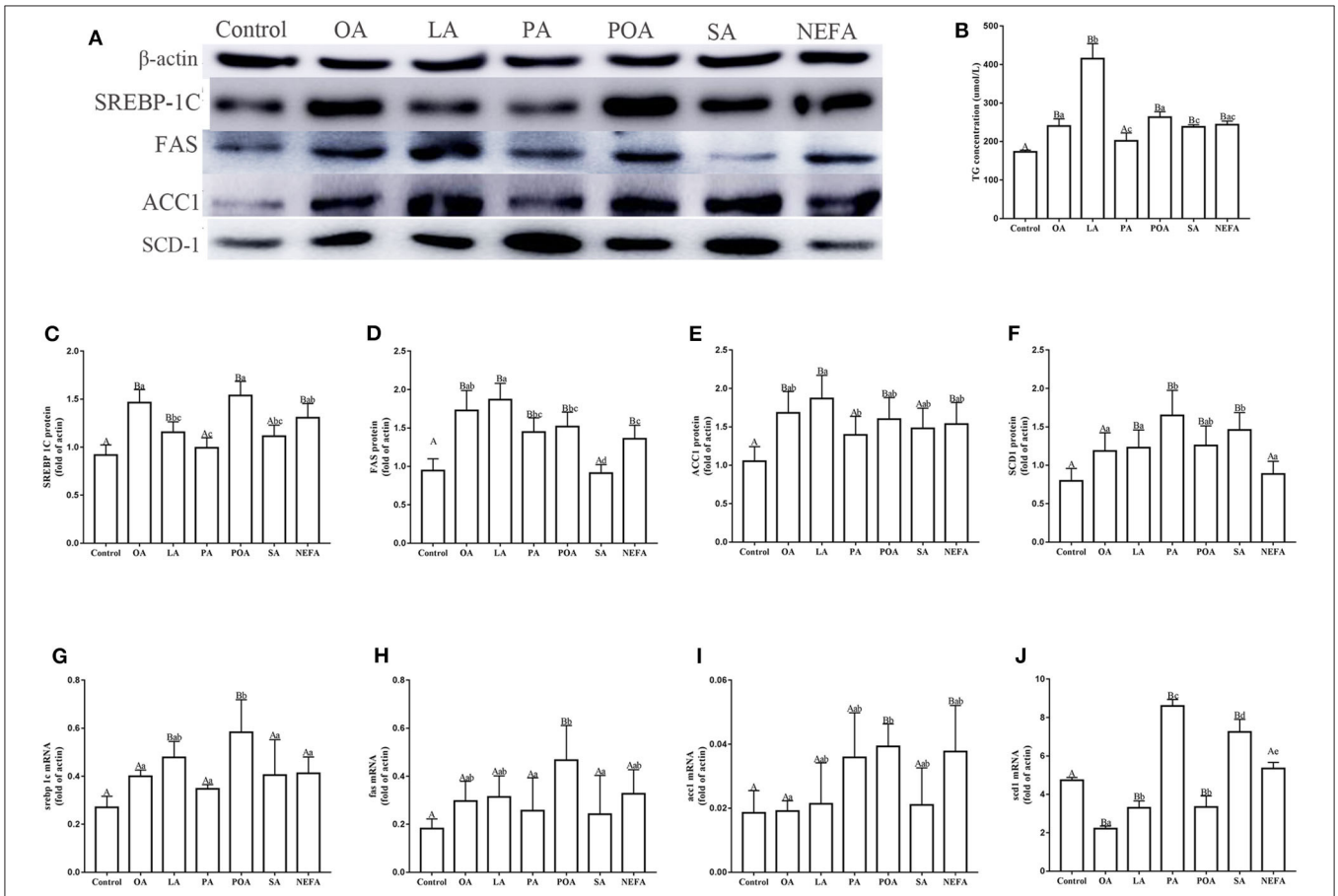

FIGURE 2 | Effects of fatty acids on fat synthesis. Cells were treated with 1.2 m*M* fatty acids for 12 h ($n = 3$ ell preparations). **(A)** β-actin, SREBP-1c, FAS, ACC1, and SCD1 protein expression were assayed by western blotting. Gray-shaded bars represent the amount (%) of β-actin, SREBP-1c, FAS, ACC1, and SCD1 in primary hepatocytes. **(B)** TG concentration was determined by ELISA. **(C–F)** SREBP-1c, FAS, ACC1, and SCD1 protein expression in primary hepatocytes were assayed by western blotting and reported relative to β-actin. **(G–J)** SREBP-1c, FAS, ACC1 and SCD1 mRNA expression were assayed by real-time RT-qPCR. Data are means ± SEM of individual assays. Each assay was performed in triplicate. Uppercase letter vs. control $p \leq 0.05$, lowercase letter vs. fatty acid group $p \leq 0.05$, the same letter vs. no letter mark $p > 0.05$.

solution contained OA (22.9 mM), LA (2.6 mM), PA (16.8 mM), SA (7.6 mM), and POA (2.8 mM). The concentration of OA, LA, POA, PA and SA was 50 mM after dissolution with 0.1 *M* KOH, and stored at −20°C after filtration and sterilization. Fatty acids were diluted to 1.2 mM in RPMI-1640 basic medium and added to the 6-well tissue culture plate after 12 h of starvation for 12 h.

RNA Extraction and PCR

Total liver RNA was extracted using TRIZOL (Invitrogen Corporation, Carlsbad, CA, USA). Total RNA was dissolved in diethylpyrocarbonate water. The RNA was transcribed into cDNA by an oligonucleotide primer using Reverse Transcriptase M-MLV (RNase H-). *GRP78*, *SREBP-1c*, *FAS*, *SORT1*, *ACC1*, *ApoB100*, and *SCD1* mRNA expression were assayed with an Applied Biosystems 7300 real-time PCR system using SYBR Premix Ex Taq I (F. Hoffmann-La Roche AG, Basel, Switzerland). Gene primers were designed using Applied Biosystems Primer Express software and are shown in **Table 1**.

Western Blot Assay

Hepatocytes were harvested and washed twice in ice-cold PBS. Total cellular protein was extracted using a protein extraction kit according to the manufacturer's instructions. Aliquots of 60 μg total protein were denatured for 5 min at 100°C and loaded on 10% sodium dodecyl sulfate polyacrylamide gels and electro-transferred onto polyvinylidene difluoride (**PVDF**) membranes. After incubation with blocking buffer, membranes were exposed to polyclonal GRP78 (78 kD, 1:250, sc-376768; Santa Cruz, CA), SREBP-1c (68 kD, 1:1000; NB100-2215; Novus, USA), FAS (273 kD, 1:1000; C2065; Cell Signaling, Danvers, MA, USA), SORT1 (85 kD,1:1000; ab16640, Abcam, Cambridge, MA), P65 (65 kD, 1:1000; D14E12; Cell Signaling; Danvers, MA, USA), ACC1 (265 kD, 1:1000; ab45174, Abcam, Cambridge, MA), CHOP (27 kD, 1:1000; L63F7; Cell Signaling, Danvers, MA, USA), PCSK9 (65–80 kD, 1:1000; 85813, Cell Signaling; Danvers, MA, USA), SCD1 (37 kD, Cell Signaling; Danvers, MA, USA). After washing and incubation with secondary antibody, immunoreactive bands were detected by enhanced chemiluminescence (Beyotime, China) solution. The imprinting was exposed with X-ray film to radiograph the spectral band, and the gray value of the spectral band was measured with bandscan software version 5.0 (prozyme Inc, San Leandro, CA). Protein abundance is reported relative to that of β-actin as a ratio of optical densities.

Detection of Biochemical and Oxidative Stress Indices

Triacylglycerol content in cells was detected by TG test kit (Mnzyme method, Solarbio Life Sciences, Beijing, China), H_2O_2 and malonaldehyde (**MDA**) in cells were detected by H_2O_2 Assay Kit and Lipid Peroxidation MDA Assay Kit (Beyotime Biotechnology, Shanghai, China).

Detection of Very Low Density Lipoprotein (VLDL) Content

The content of VLDL in the cells was detected by the Cattle Low Density Lipoprotein Assay Kit (Lengton Bioscience Co, Shanghai, China). Refer to the instructions of the kit for specific steps.

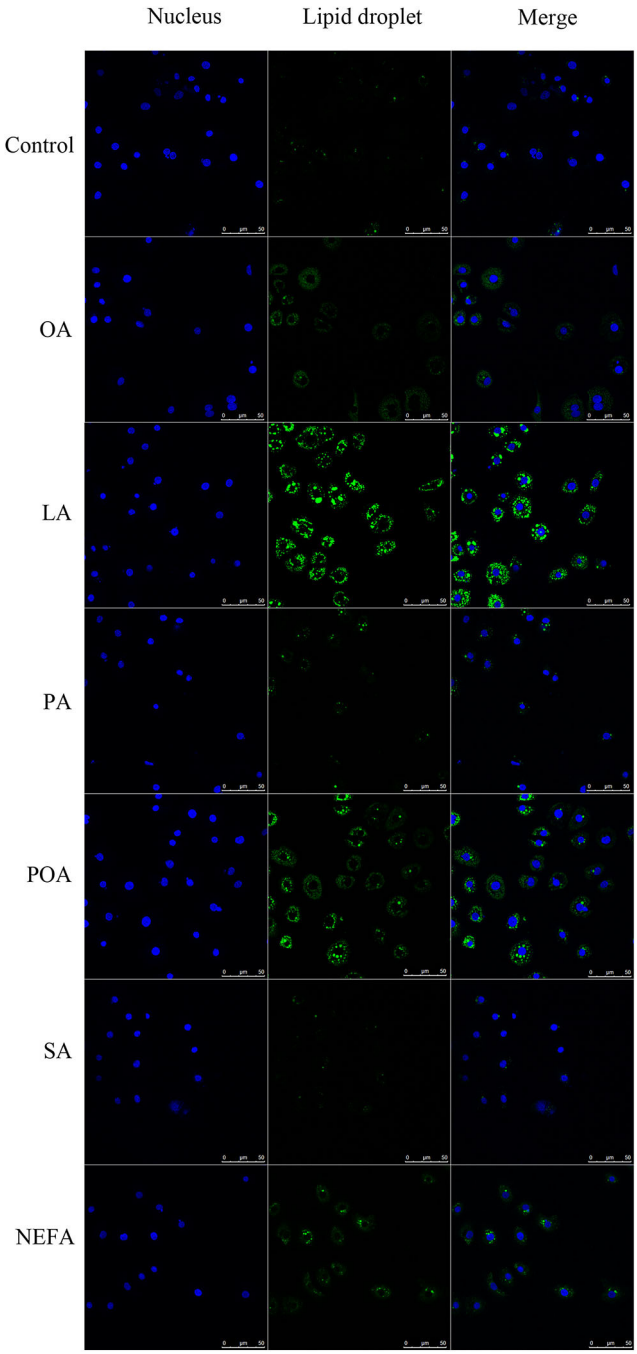

FIGURE 3 | Effects of fatty acids on the formation of lipid droplets. Cells were treated with 1.2 mM fatty acids s for 12 h, then lipid droplets in cells were stained by BODIPY, and cell nuclei were stained by Hoechst. Blue fluorescence indicates cell nuclei; green fluorescence indicates lipid droplets, the intensity of green fluorescence increases with the number of lipid droplets in the cell.

Confocal Microscopy of Lipid Droplets

Cells were inoculated into a 12-well culture plate with about 5,000 cells per well. A 1 mL cell suspension was added to each well. After 24 h of culture, the medium was discarded, and 1 mL of serum-free and double antibody-free medium was added for 12 h. Cells were stimulated with 1.2 m*M* fatty acid for 12 h. Subsequently, cells were cleaned twice with phosphate-buffered saline (**PBS**) and fixed for 30 min with 4% paraformaldehyde. After cleaning with PBS for three times, BODIPY 493/503 dye was added for 30 min in the dark. Cells were washed twice with PBS, the nuclei were then stained with Hoechst (Beyotime Biotechnology, Shanghai, China) for 8 min prior to fluorescent microscopy.

Reactive Oxygen Species (ROS) Determination

Dilute DCFH-DA in DMEM medium to a final concentration of 10 μm. After treating the cells, wash them twice with PBS, collect the cells in a 15 ml centrifuge tube by trypsinization. Mitochondrial ROS was assayed by labeling cells with 10 m*M* dihydrorhodamine 123 (DHR123, Molecular Probes) for 15 min. Samples were assayed by FACS using a Canto IITM cell sorter (BD Biosciences, New Jersey, USA). The results were analyzed using Cell Quest software (BD Biosciences, New Jersey, USA).

Statistical Analysis

The experiments were conducted in 4 separate cell preparations from four calves using three replicates per treatment. Data were analyzed using SPSS 22.0 (SPSS Inc., Chicago, IL) and GraphPad Prism 7.00 (GraphPadSoftware, San Diego, CA). Results are reported as means ± standard error of the mean (SEM). Statistical significance was determined using two-tailed unpaired Student's *t*-test or one-way ANOVA followed by Tukey's *post-test* for comparisons between 2 or among more than 2 groups, respectively. Differences were considered significant at $P < 0.05$.

RESULTS

Effects of Different Fatty Acids on Oxidative Stress and ER Stress in Primary Calf Hepatocytes

The protein abundance of NF-κB (p65) in fatty acid-treated cells increased significantly (**Figure 1A**; $P \leq 0.05$), suggesting an inflammatory response. The GRP78 and CHOP protein abundance in the fatty acid-treated groups was greater than those in the control group. CHOP protein abundance in the PA, SA and NEFA groups was greater than those of other groups, and GRP78 mRNA abundance in the PA and SA groups was greater than those of other groups (**Figures 1A–D,F,G**; $P \leq 0.05$).

Fatty acid challenge promoted generation of oxidative stress. The content of H_2O_2 in the single fatty acid-treated groups was greater than that of the control group, and the content in the PA and SA group was greater compared with other groups (**Figure 1E**). The MDA content in OA, PA, POA, SA, and NEFA groups was greater than the control and LA groups (**Figure 1H**). The ROS content in the fatty acid-treated groups was greater compared with the control group (**Figure 1I**).

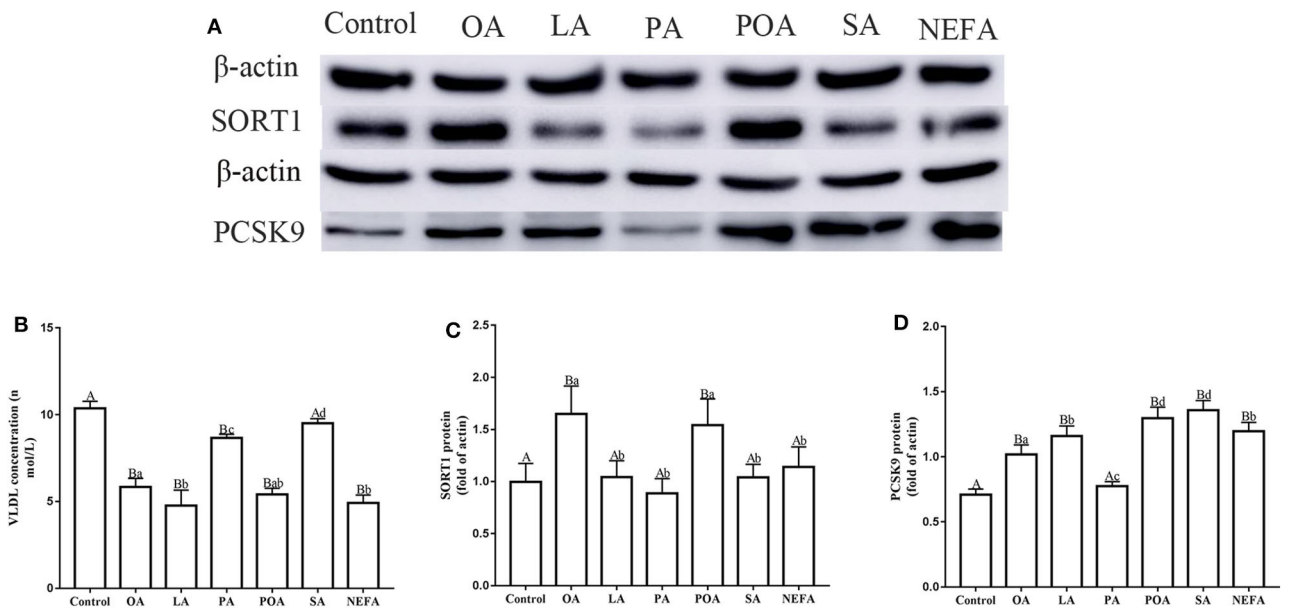

FIGURE 4 | Effects of different fatty acids on the assembly and synthesis of VLDL in primary hepatocytes. Cells were treated with 1.2 m*M* fatty acids for 12 h ($n = 3$ ell preparations). **(A)** β-actin, SORT1, and PCSK9 protein expression were assayed by western blotting. Gray-shaded bars represent the amount (%) of β-actin, SORT1, and PCSK9 protein in primary hepatocytes. **(B)** VLDL concentration was determined by ELISA. **(C,D)** SORT1 and PCSK9 expression were reported relative to that of β-actin. Data are means ± SEM. Each assay was performed in triplicate. Uppercase letter vs. control $p \leq 0.05$, lowercase letter vs. fatty acid group $p \leq 0.05$, and same letter vs. no letter mark $p \geq 0.05$.

Effects of Fatty Acids on TG Synthesis

Protein abundance of SREBP-1c in OA, LA, POA and NEFA groups was greater than the control group; the protein abundance of SREBP-1c in OA group and POA group was significantly higher than that in NEFA group (**Figures 2A,C**; $P < 0.05$). The mRNA abundance of SREBP-1c in the LA and POA groups was greater compared with other groups (**Figure 2G**; $P \leq 0.05$). Compared with the control, protein abundance of FAS with OA, LA, PA, POA, and NEFA groups was greater overall. In addition, FAS protein abundance with OA and LA was greater compared with the NEFA group; abundance of FAS mRNA with POA was greater compared with other groups (**Figures 2D,H**; $P \leq 0.05$). Protein abundance of ACC1 with OA, LA, POA, and NEFA was greater compared with the control group. Abundance of ACC1 mRNA with POA and NEFA was greater compared with other groups (**Figures 2E,I**, $P \leq 0.05$).

The protein abundance of SCD1 with LA, PA, POA, and SA was greater compared with the control group. In addition, protein and mRNA abundance of SCD1 with PA and SA was greater compared with the NEFA group ($P \leq 0.05$). Abundance of SCD1 mRNA with OA, LA, and POA was lower compared with both the PA and SA groups (**Figures 2F,J**). Content of TG with OA, LA, POA, SA, and NEFA groups was greater compared with the control group. Further, TG content with LA was greater compared with other fatty acid groups (**Figure 2B**).

Effects of Different Fatty Acids on Lipid Deposition of Primary Calf Hepatocytes

The amount of lipid droplets produced in response to NEFA or individual fatty acids was greater compared with the control group. Further, lipid droplets produced with LA were greater compared with other groups. The amount of lipid droplets produced with OA, LA, POA, and NEFA was greater compared with other groups (**Figure 3**).

Assembly and Synthesis of VLDL

Except for SA, concentration of VLDL in the fatty acid-challenged groups was lower compared with the control group (**Figure 4B**). Protein abundance of SORT1 with OA and POA was greater compared with other groups (**Figures 4A,C**). Protein abundance of PCSK9 with OA, LA, POA, SA, and NEFA was greater compared with the control group (**Figures 4A,D**). Fluorescence intensity of ApoB100 with fatty acid addition group was lower compared with the control group, with the response in the PA and SA groups being lower than other fatty acids (**Figure 5**).

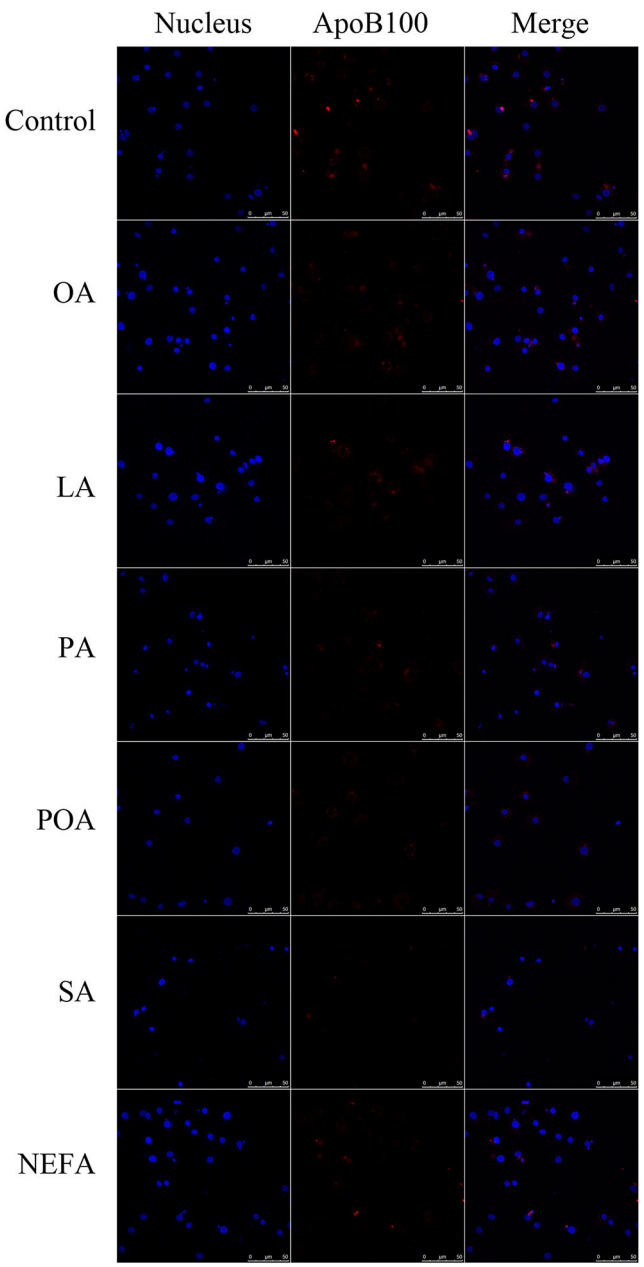

FIGURE 5 | The effect of fatty acids on the expression of ApoB100 in primary hepatocytes. Cells were treated with 1.2 m*M* fatty acids for 12 h. The ApoB100 in cells were stained by BODIPY, and cell nuclei were stained by Hoechst. Blue fluorescence indicates cell nuclei; red fluorescence indicates ApoB100, the intensity of red fluorescence increases with the expression of ApoB100 in the cell.

DISCUSSION

In dairy cows, fatty liver is characterized by high plasma NEFA concentrations and occurs most frequently during periods of NEB. High influx of NEFA into liver could promote oxidative stress, altered lipid metabolism (26), and hepatic steatosis (27). *In vitro* studies with HepG2, HuH7, and WRL68 hepatocytes indicated that elevated supply of exogenous oleic acid and palmitic acid caused hepatic steatosis and hepatocyte damage (24). However, a correlation between specific plasma fatty acids and liver damage during early lactation in dairy cows is unclear. Therefore, in our study, the characteristics of the most common NEFA in blood plasma (POA, SA, OA, and LA) induced lipid deposition and injury in primary calf hepatocytes was analyzed.

Overall, the SFA used in the present study led to greater abundance of the ER stress markers GRP78 and CHOP,

oxidative stress markers H_2O_2 and MDA, and the inflammation transcription factor NF-κB subunit p65 suggesting the induction of severe hepatocyte damage. Endoplasmic reticulum stress is caused by accumulation of misfolded proteins (28). In the process of protein folding, ROS are produced as by-products, which leads to alterations in redox balance and oxidative stress (29). NF-κB is a transcription factor that regulates gene expression and can affect cell survival, growth, proliferation, and apoptosis. Its signaling plays a key role in NAFLD (30). Persistent oxidative stress and ER stress activates apoptotic pathways and triggers hepatocyte damage (31). *In vivo*, the existence of ER stress in the liver of severe fatty liver cows may presage its participation in fatty liver progression in dairy cows (4).

Mitochondrial activities, including beta-oxidation of free fatty acids, production of ATP, and ROS are all central to homeostatic regulation of lipid and energy metabolism in hepatocytes (32). In addition, compounds such as ROS are important for the regulation of apoptosis (33). *In vitro*, compared with OA, exogenous PA led to greater ROS accumulation in rat hepatoma cells, a response partly caused by the inability of mitochondria to handle large amounts of exogenous long-chain fatty acids, which often leads to mitochondrial dysfunction (34, 35). The content of MDA produced by lipid peroxidation by ROS is also a marker for oxidative stress, thus, the greater concentration of MDA in response to exogenous SFA in the present study underscored the existence of serious oxidative stress. *In vivo* study, mid-lactating cows was supplemented SPA, α-linolenic acid, and conjugated linoleic acid for 6 weeks, respectively, it was found that SFA supplemented cows showed more serious oxidative stress with a lower plasma glutathione peroxidase activity than in α-linolenic acid, and lower plasma concentration of β-carotene than in α-linolenic acid and conjugated linoleic acid (36). Our previous *in vitro* studies confirmed that increases in ROS during mitochondrial metabolism can further enhance ER stress (37).

The greater content of lipid droplets and triacylglycerol in response to UFA compared with SFA along with the greater abundance of SREBP-1c, FAS, and ACC1 suggested a potential protective role by channeling utilization of fatty acids for TG synthesis to help reduce lipid toxicity (38). These responses agree with previous data indicating that exogenous USFA can activate SREBP-1c transcription and upregulate FAS and ACC1 (39). From a mechanistic standpoint, this effect of exogenous UFA might involve loss of torsinA activity, which was shown to control budding of lipid droplets from the ER and, thus, controlling availability of fatty acids to mitochondria and the rates of fatty acid oxidation (40). Future studies exploring the role of lipid droplet-associated proteins in the control of mitochondrial fatty acid metabolism appear warranted (39).

The VLDL is essential for export of endogenous TG out of the liver, and the lower concentration of VLDL observed primarily with USFA and NEFA suggested a potentially negative

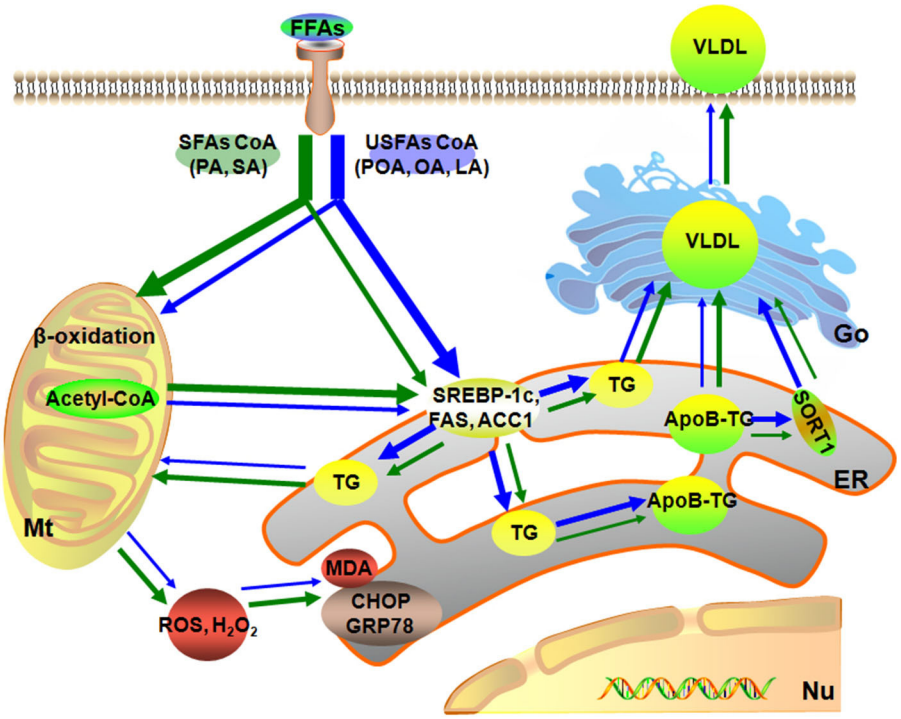

FIGURE 6 | Model of different fatty acids induced lipid accumulation and liver injury characteristics of primary calf hepatocytes. The green arrow represents the SFAs metabolic, blue arrow represents the USFAs metabolic. The thickness of the arrow represents the level of fatty acid metabolism. Mt, mitochondria; Nu, nucleus; ER, endoplasmic reticulum.

impact on the export process. This idea is supported by the greater abundance of ApoB100 and SORT1 coupled with greater concentration of TG. ApoB100 is essential for VLDL assembly, aiding in the "lipidation" of nascent VLDL through a concerted mechanism with microsomal triglyceride transfer protein (**MTP**) in the lumen of the ER. SORT1 is an intracellular sorting receptor for ApoB100. It interacts with ApoB100 in the Golgi and facilitates the formation and hepatic export of ApoB100-containing lipoproteins (41). From the present data, it is possible that the elevated concentration of exogenous fatty acids overwhelmed the VLDL synthesis machinery, which agrees with a previous study demonstrating changes in ApoB100 secretion as a function of fatty acid availability (42). In that context, we speculate that the greater abundance of PCSK9 in response to exogenous USFA and SFA (except SA) was a response to counteract the impairment in VLDL export. In mice, PCSK9 interacts directly with cytoplasmic ApoB100 and prevents its degradation via the autophagosome/lysosome pathway (43). Thus, it is possible that abundance of PCSK9 is another component of the mechanisms that help control liver fat accumulation. However, *in vivo* study found that feeding LA and linolenic acids as unprotected oilseeds increased dry matter intake over time at a greater extent for cows fed PA, improved the energy status, and lowered hepatic lipids and triacylglycerol contents, which may contribute to enhance the health status of transition dairy cows (44).

In summary, a dose of 1.2 mmol/L of individual or mixture of fatty acids induced different degrees of lipid accumulation and hepatocyte damage. Compared with USFA (POA, OA, and LA), exogenous SFA (PA and SA) led to more pronounced oxidative and ER stress. In contrast, exogenous USFA led to greater TG accumulation driven in part by an induction of lipogenesis along with ApoB100 and SORT1. That effect, however, was associated with lower VLDL discharge into cell culture medium (**Figure 6**). Overall, the data suggest that type of exogenous long-chain fatty acid can induce distinct effects at the level of the hepatocyte. Although many published studies using hepatocytes from calves to address metabolism of the adult ruminant liver (45–47), whether these responses could be induced *in vivo* by feeding specific fatty acids remained to be determined.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee for the Use and Care of Animals, Heilongjiang Bayi Agricultural University.

AUTHOR CONTRIBUTIONS

BZ, WY, and CXu designed this project. RL, SW, ZD, YZ, and XM performed the experiments and analyzed the results. JL and CXi revised the manuscript. BZ and WY drafted this manuscript. All authors revised final version of the manuscript.

FUNDING

This work was supported by the National Program on Key Research Project of China (Project No. 2018YFD0501702); Young Innovative Talents Training Program of Ordinary Undergraduate Universities in Heilongjiang Province (No. UNPYSCT-2018081).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 15 October 2020
doi: 10.3389/fvets.2020.556764

Increasing the Sustainability of Maize Grain Production by Using Arbuscular Mycorrhizal Fungi Does Not Affect the Rumen of Dairy Cattle (*Bos taurus*) and Buffalo (*Bubalus bubalis*)

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OPEN ACCESS

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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: 28 April 2020
Accepted: 31 August 2020
Published: 15 October 2020

Citation:
Chiariotti A, Edwards JE, Hermes GDA, Catillo G, Meo Zilio D, Di Giovanni S, Smidt H and Buttazzoni L (2020) Increasing the Sustainability of Maize Grain Production by Using Arbuscular Mycorrhizal Fungi Does Not Affect the Rumen of Dairy Cattle (Bos taurus) and Buffalo (Bubalus bubalis). Front. Vet. Sci. 7:556764. doi: 10.3389/fvets.2020.556764

New approaches are needed to improve the sustainability of feed production and utilization by ruminants. Promising approaches include increased use of buffaloes for more sustainable milk production, and arbuscular mycorrhizal fungi (AMF) to reduce crop production input needs. However, studies assessing the effect of crops grown in the presence of AMF on rumen microbial utilization are limited. Based on current knowledge, we hypothesized that maize grain grown on AMF-inoculated soil affected ruminal fermentation and microbiota, and that this effect differed between buffalo and cattle. A dietary cross-over study (four weeks per diet) was conducted using rumen-cannulated cattle ($n = 5$) and buffalo ($n = 6$) to assess the effect of maize grain (3.9% (w/v) of diet) grown on soil with or without AMF (15 kg/ha) on ruminal fermentation and microbiota. Production of maize on AMF-treated soil did not affect any of the assessed ruminal fermentation parameters, microbial concentrations, or prokaryotic community composition (using prokaryotic 16S rRNA gene sequence analysis). In contrast, host type had numerous effects. Protozoal counts, lactate, total VFA and isobutyrate, were significantly higher in buffaloes compared to cattle. Conversely, butyrate was significantly lower in buffaloes than in cattle. Host type explained 9.3% of the total variation in prokaryotic community composition, and relative abundance of nine amplicon sequence variants significantly differed between host types. These findings indicate that AMF treatment of maize crops has no detrimental impact on the value of the resulting maize grains as a ruminant feed, and provides additional insight into rumen-based differences between cattle and buffalo.

Keywords: rumen microbiome, bacteria, archaea, protozoa, anaerobic fungi, fermentation

INTRODUCTION

Increasing world population, urbanization, and the growing concern over the environmental impact of animal farming means a long-term global strategy for more intensive and sustainable ruminant production is needed (1). Ruminant livestock are important not only for the production of high quantity and quality animal protein (milk and meat) in human diets, but also their ability to produce this using fibrous feeds that cannot be used by humans (2). Furthermore, low-quality feedstuffs, which contain high levels of fiber and low levels of fermentable carbohydrates, are more efficiently utilized by buffaloes than cows. As such, there is increasing interest in this more sustainable ruminant species for livestock production. This is evidenced by the 15.7% increase in buffalo milk production worldwide compared to 4.09% in dairy cows during 2014–2018 (FAOstat data). In Italy, the increase in the same period was up to 21%. This is because in Italy, buffalo milk is used to produce mozzarella cheese which is the third largest Italian DOP (i.e., protected designation of origin) cheese in terms of market value.

Buffalo are more efficient at utilizing low-quality feedstuffs, compared to cattle, due to differences in their digestive system anatomy and physiology (3). Compared to cattle, buffalo have higher rumen retention time (4) and higher feed digestibility (5–7). Associated with this, rumen microbial concentrations and community composition have also been shown to differ between cattle and buffalo (7–9). In order to decrease the impact of both buffalo and cattle production on the environment, new approaches are also needed to improve the sustainability of feed production, for example by decreasing the need for crop fertilization, pest and weed control, and water input. Mycorrhizal fungi are one means of doing this, as they can decrease crop production costs and associated environmental pollution risk.

Mycorrhizal associations of fungi with plants are present in almost all ecosystems, from deserts to arable land (10). Arbuscular mycorrhizal fungi (AMF) are the most common type of fungi that form a symbiotic relationship with terrestrial plants (11). The mycorrhiza formed essentially result in an extension of the plant's root system (12). AMF obtain carbohydrates from their plant host, and in return they provide the plant with mineral nutrients including phosphorus, nitrogen, and potassium (11, 13). AMF positively influence the accumulation of iron and zinc in the plant, which are among the numerous minerals that are relevant to ruminant livestock nutrition (14–16).

As well as exchanging minerals for nutrients, AMF relieve plants from abiotic stress such as drought, salinity and heavy metals (17–19). Protection of host plants from biotic stresses, such as microbial pathogens, has also been reported (20, 21). Furthermore, AMF can contribute to mycotoxin control (22) and induction of the expression of defense-related genes (23). In broader terms, AMF also improve soil structure (24) and can even play an important role in maintaining plant biodiversity and ecosystem stability (25).

Some commercially available AMF preparations include other biological components, such as plant growth promoting rhizobacteria (PGPR) (26–28). PGPR are very effective in promoting plant growth by releasing growth stimulating hormones, and can also give some protection against soil-borne pathogens (29, 30). With maize, PGPR have been shown to improve the mineral status of the plant, promote mycorrhiza colonization of maize roots and increase maize growth (30, 31). As maize is a basic feed for ruminants, differences in its quality can have a significant impact on milk yield and composition. It has been reported that silages from AMF treated forage crops have a higher protein and dry matter content compared to non-AMF treated forage crops (26, 28). Inoculation with both the PGPR strain *Pseudomonas fluorescens* Pf4 and AMF has also been shown to promote maize growth under field conditions, and can differentially affect grain nutritional content (16). However, only limited studies to date have looked at the effect of AMF applied during the production of feed on livestock.

A study with dairy cows and buffalo fed silages of sorghum and maize produced in the presence of AMF looked at milk production parameters and *in vitro* feed digestibility (28). Another study assessed the effect of AMF on the *in vivo* digestibility of fresh barley and berseem clover in goats (27). Both studies found a positive effect of AMF on forage digestibility, however, neither of these studies directly assessed ruminal fermentation parameters and the associated microbiota. In a preliminary communication, we have reported on a feeding trial with lactating Holstein cows where we evaluated the effect of maize grain grown on mycorrhized soil (32). Differences were found in term of maize grain degradation characteristics and, compared to the control maize grain fed animals, the grain grown on soil inoculated with AMF increased rumen microbial concentrations, dry matter intake and milk protein content.

To date, no other information is available regarding the effect of maize grains grown on soil inoculated with AMF on the rumen microbiota and associated fermentation in cattle, and nothing is known in buffalo. Based on the available knowledge outlined above, we hypothesized that maize grain grown on soil inoculated with AMF affects ruminal fermentation and associated microbiota, and that this effect will differ between buffalo and cattle. In order to test this hypothesis, a dietary cross-over study was conducted using rumen-cannulated Holstein Friesian dairy cows and Mediterranean buffaloes. Ruminal fermentation parameters and rumen microbial concentrations were measured, and barcoded amplicon sequencing of the 16S rRNA gene was used to assess rumen prokaryotic community composition.

MATERIALS AND METHODS

Animals and Experimental Design

Rumen cannulated, non-lactating Mediterranean buffalo (*Bubalus bubalis*) cows ($n = 6$) and Holstein-Friesian (*Bos taurus*) dairy cows ($n = 6$) were used for the trial. Animals were kept in paddocks, with three animals of the same host type in each paddock. Animals were fed the same basal diet for 4 weeks prior to the start of the trial, which had a crossover design comprised of two experimental periods. Within the first experimental period, half of the animals for each host type were assigned to the diet containing maize grain produced with AMF, and the other half were assigned to the diet containing maize

grain produced without AMF. These experimental diets were then fed for 4 weeks. After this, the basal diet was fed again for 4 weeks as a "washout" period before start of the second experimental period. In the second experimental period, diets were then switched over within the same host type, and were fed for 4 weeks.

Both host type groups started the study with six animals, however, due to one Holstein-Friesian cow having to be euthanized during the first experimental period the number of cattle was subsequently decreased to $n = 5$. Rumen sampling was performed during the last 3 consecutive days of each experimental period. Statistical analysis was performed using animal replication, i.e., for cattle $n = 5$, and for buffalo $n = 6$.

The animal study research protocol was approved by the National Ethics Committee (Ministry of Health Decree 26/2014, authorization n°399/2018, Italy) in accordance with the guidelines established by the EU Council/Directives 86/609/EEC.

Maize Grains and Diet

Mycorrhized soil maize grains (M) as well as control maize grains (C), were both produced on two farms in a maize cropping area in the northern Italy near Cremona. For only the mycorrhized soil produced maize grains, Micosat ® Cereal, was applied (15 kg/ha) as a granular preparation when the maize crop was sowed according to the manufacturer's recommendations. The Micosat® Cereal product is a biostimulant microbial consortium consisting mainly of 40% AMF species (*Glomus caledonium* GM 24, *G. coronatum* GU 5, and *Rhizophagus irregularis* RI 31) in the form of ground spores, hyphae, and root fragments of donor plants, along with 9.3% (2.0×10^7 C.F.U./g) of both PGPR (*Bacillus subtilis* BR 62, *Streptomyces* sp. ST 60, *Paenibacillus durus* PD 76) and saprophytic fungi (*Trichoderma harzianum* TH 01, *Trichoderma atroviride* TA 28). The composition of the remainder of the product is abiotic (C.C.S. Aosta s.r.l., Quart, AO, Italy). The rhizobacteria and the fungi are added by the producer, as they are thought to have additional value alongside AFM.

Diets containing the maize grains (Diet M or Diet C) were fed once per day (11 am) as a total mixed ration (TMR) with the same proportion of all ingredients for both species, and each group receiving 60 kg feed/day (i.e. 20kg/head/day on average). Feed refusals were not collected (it was observed during the study that feed refusals were all generally <1kg for both host types), and consequently dry matter intake (DMI) was not assessed or predicted in this study. After the loss of the Holstein-Friesian cow from the trial, the corresponding group then received 40 kg feed/day. Details of the composition of the TMR and basal diet are provided in the **Supplementary Methods**. Dry matter and chemical analysis of the basal diet, maize grains and the associated TMR diets (**Table 1**) were performed according to the methods described in the **Supplementary Methods**. Buffalo and cattle nutritional requirements are provided in **Supplementary Tables 1** and **2**, respectively.

Rumen Sampling

Rumen digesta samples (1 L) were collected by hand from each animal 1 h before morning feeding on 3 consecutive days. Sampling was done from the dorsal and ventral sac of the rumen via the rumen cannula, and the samples pooled. Two aliquots from each rumen sample were made. The first aliquot was strained through three layers of cheesecloth and used for direct determination of pH and protozoa counts. The second aliquot was bag filtered (BagPage. Interscience, France) and treated with a homogenizer for 5 min (Stomacher, VWR International, Pennsylvania, USA). Aliquots of the homogenate were then processed as follows for later analysis: frozen at −80°C for microbial analysis, preserved with sulfuric acid and stored at −20°C for ammonia, volatile fatty acids (VFA) and lactate analysis.

Fermentation Analysis

Fermentation analysis was performed on all the rumen samples, i.e., 2 experimental periods × 11 animals (5 cows + 6 buffalo) × 3 days. Metabolic fermentation products

TABLE 1 | Dry matter (g/kg) and chemical analysis (g/kg DM) of the basal diet, maize grains and the associated total mixed ration (TMR) diets.

Item	Dry matter	Crude protein	Crude fiber	Ether extract	Starch	Ash	NDF#	ADF#	ADL#	Hemi-cellulose	Cellulose
Basal diet	609.1	95.8	187.1	22.4	170.8*	55.9	427.6	230.7	34.6	197.0	196.0
Maize grain M	881.7	89.1	26.9	36.6	696.4	12.6	158.1	39.7	16.1	118.4	23.6
Maize grain C	882.8	92.3	30.8	39.8	711.1	12.6	145.6	44.5	15.7	101.1	28.8
TMR M	556.9	157.9	213.7	33.0	298.4*	62.8	447.8	259.7	44.3	188.1	215.4
TMR C	557.6	158.9	214.3	33.5	299.0*	62.8	445.7	260.4	44.2	185.2	216.2

Maize grain M, grown on AMF treated soil.
Maize grain C, grown on untreated soil.
TMR C, diet containing maize grain grown on untreated soil.
TMR M, diet containing maize grain grown on AMF treated soil.
#*NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin.*
**Non-Structural Carbohydrates.*

(i.e. VFA and lactate) were analyzed using high-performance liquid chromatography (HPLC). The procedure is described in detail in the **Supplementary Methods**. Ammonia samples were analyzed according to the 4030-method reported in the "Analytical Methods for Water" (33).

DNA Extraction

DNA was extracted from the rumen homogenate samples using a protocol involving a combination of bead beating, Stool Transport and Recovery (STAR) buffer (Roche Diagnostics Nederland BV, Almere, The Netherlands) and the Maxwell ® 16 Instrument (Promega, Leiden, The Netherlands). The procedure is described in detail in the **Supplementary Methods**.

Microbial Concentrations

Analysis of microbial concentrations was also performed on all the rumen samples as reported for fermentation analysis. Protozoa were counted using a Fuchs-Rosenthal chamber according to the Warner procedure (34). For absolute quantification of bacteria and archaea, SYBR green based qPCR assays were performed on DNA extracts using a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories BV, Veenendaal, Netherlands) as previously described (35). All qPCR analyses were carried out in triplicate with a reaction volume of 10 μL, which contained 2 μL of 1:50 dilutions of sample DNA extracts. Standard curves (10^2-10^8 copies/μL) were generated using serial dilutions of custom synthesized DNA prepared from known 16S rRNA gene sequences of *Ruminococcus albus* and *Methanobrevibacter olleyae* (accessible under ENA accession numbers: CP002403.1 and CP014265.1, respectively).

For absolute quantification of anaerobic fungi, a 5.8S rRNA gene Taqman probe based method was used as previously described (36) with the exception that a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories BV) was used. All qPCR analyses were carried out in triplicate in a reaction volume of 10 μL, which contained 2 μL of 1:50 dilutions of sample DNA extracts. The standard curve (10^1-10^8 copies/μL) for the assay was generated using serially diluted custom synthesized DNA prepared from a known partial sequence of the *Neocallimastix* sp. CF17 *rrn* operon that contains the full 5.8S rRNA gene (accessible under ENA accession number: GU055516.1).

Prokaryotic Community Composition Analysis

Samples from the first day of rumen sampling, for each animal and period, were used for prokaryotic community composition profiling by amplification and barcoding (37) of the V4 variable region of the 16S rRNA gene using the barcoded primers 515F (Parada): 5′-GTGYCAGCMGCCGCGGTAA-3′ (38) and 806R (Apprill): 5′ GGACTACNVGGGTWTCTAAT-3′ (39).

For each sample, triplicate PCRs were performed in a volume of 50 μL containing 1× HF buffer (Finnzymes, Vantaa, Finland), 1 μL dNTP Mix (10 mM; Promega Benelux, Leiden, Netherlands), 1 U of Phusion® Hot Start II High-Fidelity DNA polymerase (Finnzymes), 500 nM of barcoded primers and 2 ng of template DNA. Cycling conditions consisted of an initial denaturation at 98°C for 30 s followed by 25 cycles of 98°C for 10 s, 56°C for 10 s, and 72°C for 10 s, and a final extension at 72°C for 7 min. PCR success and product size was assessed by agarose gel [2% (w/v)] electrophoresis with 1× SYBR® Safe (Thermo Scientific).

Triplicate reactions were pooled, purified using HighPrep™ (MagBio Europe Ltd, Kent, United Kingdom), quantified using a Qubit dsDNA BR Assay Kit (Thermo Scientific) and subsequently mixed in equimolar amounts. In order to assess the resulting data quality and potential effects of technical biases, a non-template control (NTC) reaction and a defined synthetic mock community of known composition (37) were included in the equimolar pool. The resulting library was sent for adaptor ligation and 150 nt paired-end sequencing on an Illumina HiSeq4000 instrument (GATC-Biotech, Konstanz, Germany, now part of Eurofins Genomics Germany GmbH).

The resulting raw data was processed with NG-Tax 2.0 using default settings (40). The processing was performed as follows. Paired-end libraries were demultiplexed using read pairs with valid and perfectly matching barcodes. Amplicon sequence variants (ASVs) were then picked. ASVs are defined as individual sequence variants, rather than a cluster of sequence variants with a shared similarity above a user specified threshold as was traditionally the case for operational taxonomic units. ASV selection used an open reference approach as follows. Sequences were ordered by relative abundance for each sample independently, and sequences were considered valid when their relative abundance was >0.1%. Initially discarded low-abundance sequences were then added to ASVs in the dataset, allowing for a single nucleotide mismatch. Taxonomy was assigned using the SILVA 16S rRNA gene reference database version 132 (41). The raw sequence data generated in this study is deposited in ENA under study accession number PRJEB37928, with individual sample accession numbers ranging from ERS4525212-ERS4525234.

Statistical Analysis

The microbial concentration and fermentation data were analyzed using the PROC GLM procedure of SAS 9.4 software (42) using the following univariate linear effects models: $Y_{ijkml} = \mu + A_i + B_j + C_k + (AB)_{ij} + a_m + e_{ijkml}$ where: Y is the observation vector for the all traits; μ is total average for the trait; A_i is the fixed effect of species: buffalo or cattle; B_j is the fixed effect of diet: M or C; C_k is the fixed experimental period $(AB)_{ij}$ is the interaction between species and diet; a_m is the animal modeled as random effect; e_{ijkl} is the random residual. The statistical significance of all traits and least-squares means were determined using Student's *t*-test in the GLM procedure. A probability value <0.05 was considered significant.

Prokaryotic community composition data was analyzed in R version 3.4.0 (43). Stacked bar graphs of individual microbiota composition were created by summarizing the relative abundance of all ASVs to either the phylum level, or to the top 15 most abundant prokaryotic genus level groupings. A stacked bar graph of the average microbiota composition of the treatment groups was also created using the summarized relative abundance of all ASVs to the top 20 most abundant prokaryotic genus level groupings. In terms of both genus level graphs,

for clarity all other genus level groupings were summarized as "Other." Pairwise distance between microbiota samples (beta-diversity) was visualized with Non-metric Multidimensional Scaling (NMDS). The NMDS algorithm places each sample into a two-dimensional space such that the between-sample distances are preserved as well as possible. The weighted UniFrac and unweighted UniFrac metrics, which are based on the phylogenetic relatedness of the ASVs, were used as estimator of microbial beta diversity between the animals. While weighted UniFrac considers the abundance of each ASV, unweighted UniFrac provides equal weight to all ASVs, thereby giving equal importance to presence or absence of low and high abundance ASVs. Redundancy Analysis (RDA) was performed to determine the multivariate effects of the environmental variables on the microbiota composition using the *rda* function from the *vegan* package (44). RDA is a gradient technique summarizing the linear relationships between a set of variables i.e., prokaryotic community composition and a set of explanatory variables such as host type, dietary intervention, and experimental period. ASV abundance was normalized by centered log ratio scaling to account for compositionality of the data (45). To determine the effect sizes and overlap of the significant environmental variables, variation partitioning was performed using the *varpart* function from *vegan*.

RESULTS

Ruminal Fermentation and Microbial Concentrations

For all of the analyzed ruminal fermentation parameters there was no significant effect of diet (**Table 2**). However, some of the ruminal fermentation parameters were affected by host type (**Table 2**). Lactate concentrations were significantly ($P = 0.04$) higher in buffaloes compared to cattle. Total VFA concentrations showed the same trend, being significantly ($P < 0.001$) higher in buffaloes compared to cattle. The molar proportion of isobutyrate was also significantly ($P = 0.02$) higher in buffaloes compared to cattle. In contrast, the molar proportion of butyrate was significantly ($P = 0.02$) lower in buffalo compared to cattle. No other ruminal fermentation parameters were significantly affected by host type (**Table 2**).

As with the ruminal fermentation parameters, diet had no significant effect on any of the analyzed rumen microbial concentrations or the archaea/bacteria ratio (**Table 2**). Host type had a significant ($P = 0.01$) effect on protozoal counts, being higher in buffaloes compared to cattle (**Table 2**). No other microbial concentrations or ratios were affected by host type (**Table 2**).

TABLE 2 | Effect of diet and host type on ruminal fermentation parameters and microbial concentrations.

	Diet[§]		Host			*P*-values	
	C	M	Cattle	Buffalo	MSE*	Diet	Host
pH	6.5	6.4	6.5	6.4	0.37	0.30	0.97
Ammonia (mg/dl)	30.6	29.9	28.9	31.6	0.09	0.76	0.25
Lactate (mM)	0.2	0.2	0.1	0.3	0.33	0.65	0.04
Total VFA (mM)	117.3	123.2	88.7	151.8	50.28	0.65	<0.001
Molar proportions:							
Acetate	66.3	65.5	63.0	68.8	12.34	0.82	0.07
Propionate	14.9	17.7	16.0	16.6	6.89	0.12	0.77
Isobutyrate	1.1	1.3	0.5	1.9	2.41	0.77	0.02
Butyrate	10.4	11.7	12.6	9.6	4.92	0.31	0.02
Isovalerate	2.3	2.4	2.3	2.3	1.23	0.89	0.96
Valerate	0.6	1.3	1.3	0.7	1.88	0.13	0.17
Acetate/propionate	4.3	4.4	4.3	4.4	1.60	0.87	0.73
Bacteria[#]	11.0	10.9	10.9	11.0	0.10	0.27	0.40
Anaerobic Fungi[#]	9.1	8.7	8.9	8.9	1.18	0.27	0.87
Archaea[#]	10.4	10.3	10.3	10.4	0.11	0.21	0.12
Protozoa[$]	4.0	4.4	3.4	4.9	2.22	0.52	0.01
Archaea:bacteria	0.9	0.9	0.9	0.9	0.01	0.72	0.34

[§]*Diet C contained maize grain grown on untreated soil. and Diet M contained maize grain grown on AMF treated soil.*
[*]*Mean Standard Error.*
[#]*Values are expressed as Log10 gene copies per g rumen sample. For bacteria and archaea this is the 16S rRNA gene. and for anaerobic fungi the 5.8S rRNA gene.*
[$]*Protozoa cell counts are expressed as* $\times 10^5$ *cells/ml filtered rumen sample.*

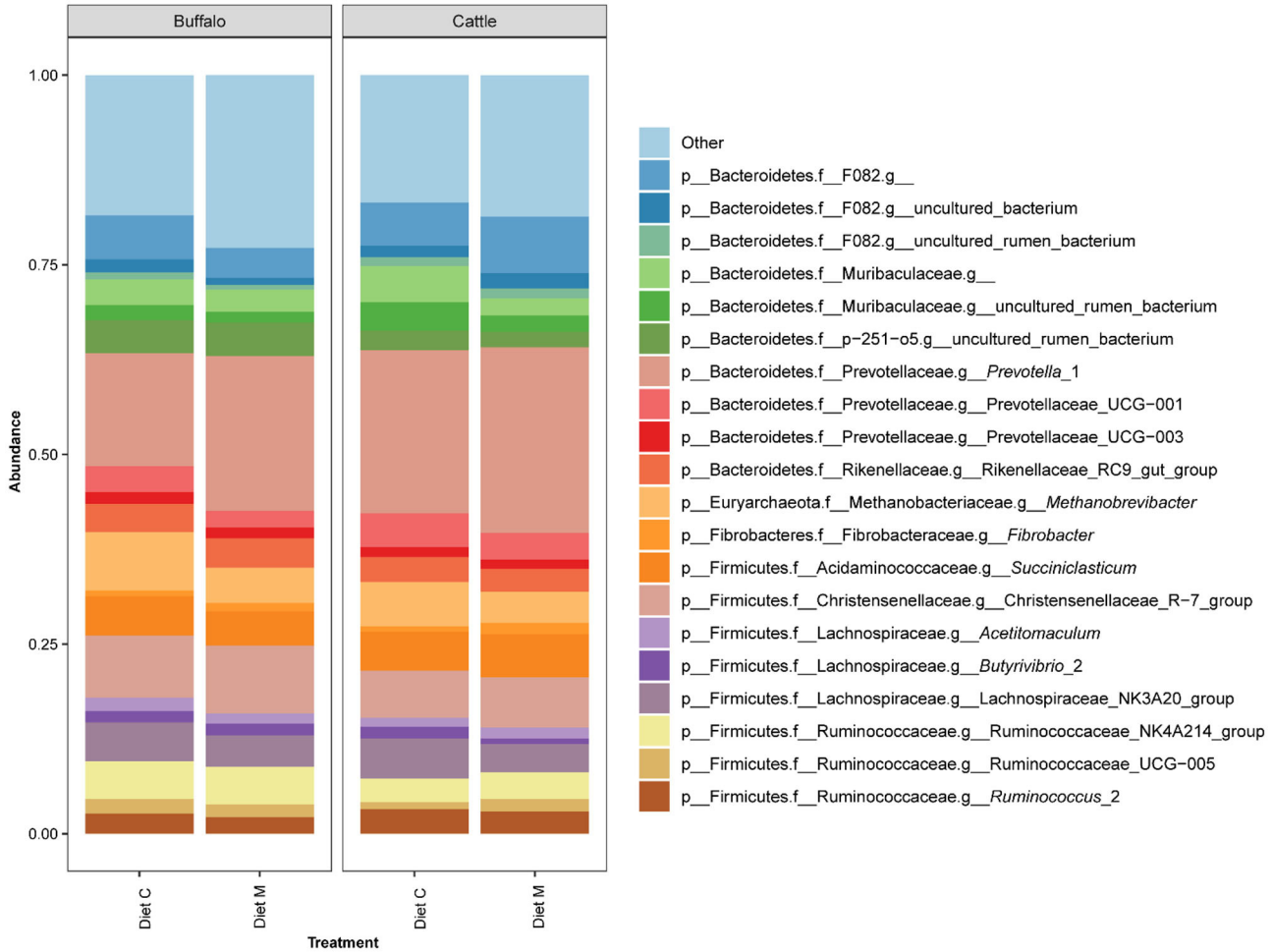

FIGURE 1 | Bar graph showing the average of the 20 most abundant genus level groupings of ASVs from the rumen microbiota of Holstein-Friesian cattle (Cattle) and Mediterranean buffalo (Buffalo) fed diets that differed in terms of containing maize grain which was grown on soil that was treated (Diet M) or not (Diet C) with a commercial AMF preparation.

Prokaryotic Community Composition

Of the 960 bacterial and archaeal ASVs detected in the dataset, only three could not be annotated by version 132 of the SILVA database. The number of ASVs annotated at the different taxonomic ranks were as follows: kingdom, 957; phylum 957; class 956; order, 955; family 945; genus 840. The two bacterial phyla Bacteroides and Firmicutes accounted for the majority of the prokaryotic community composition at the phylum level (**Supplementary Figure 1**). The 20 most predominant (>1.5% mean abundance) genus level groupings of the ASVs in the dataset belonged to the phyla Firmicutes, Bacteroidetes, Fibrobacteres, and Euryarchaeota (**Figure 1**). Among these, genus level groups within the Firmicutes were annotated as belonging to *Succiniclasticum*, *Acetitomaculum*, *Butyrivibrio* 2, Christensenellaceae R−7 group, Lachnospiraceae NK3A20 group, Ruminococcaceae NK4A214 group, Ruminococcaceae UCG−005 and *Ruminococcus* 2. Those within the Bacteroidetes were annotated as belonging to *Prevotella* 1, Prevotellaceae UCG−001, Prevotellaceae UCG−003, Rikenellaceae RC9 gut group and to families F082, Muribaculaceae and p−251−o5. Within the phyla Fibrobacteres and Euryarchaeota, only the genera *Fibrobacter* and *Methanobrevibacter*, respectively, were represented within the top 20 genus level groupings. Within each treatment group, variation between individual animals was observed at both the phylum and genus levels (**Supplementary Figures 1, 2**).

Weighted and unweighted analysis of beta diversity using unsupervised NMDS showed no separation of samples by diet, and only separation of host type in the unweighted analysis (**Supplementary Figure 3**). This indicated that differences between host types was largely due to low abundance taxa unique to one host type.

Redundancy analysis (RDA) was performed with ASV level data (i.e., not grouped at the genus level) (**Figure 2**) to determine the multivariate effects of the experiment on rumen prokaryotic community composition. In contrast to diet (*P*

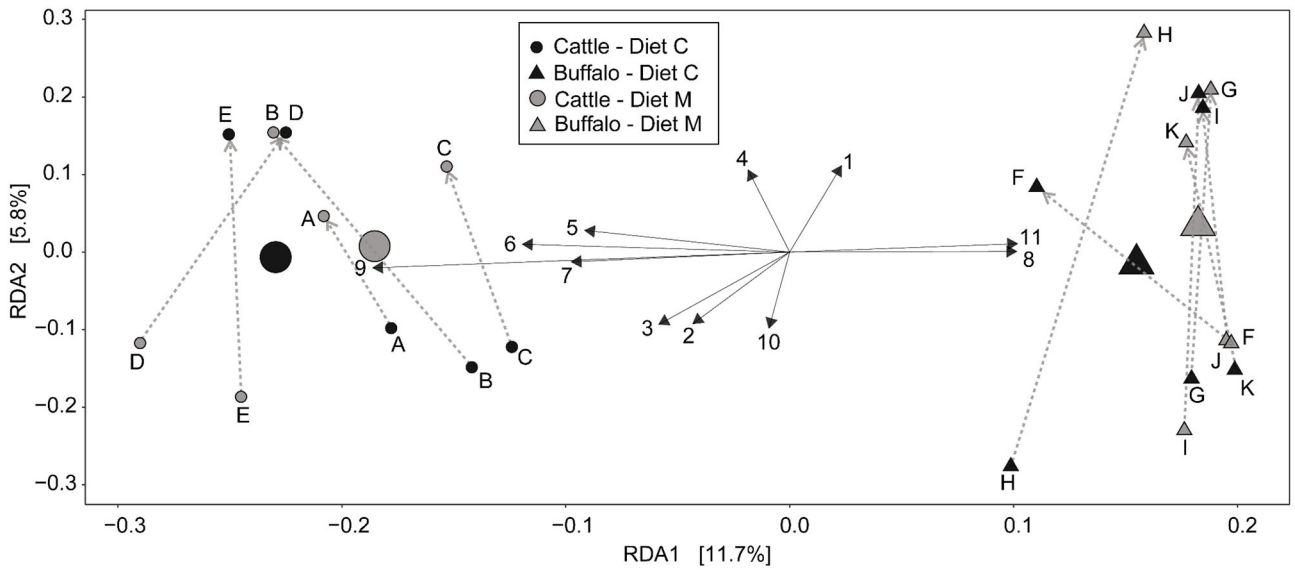

FIGURE 2 | Redundancy analysis biplot of the rumen prokaryotic communities at the level of individual ASVs. constrained by host type (cattle or buffalo) and experimental period. Small symbols are data points represent of rumen microbial communities of individual samples and are labeled with the respective animal codes for the Holstein-Friesian cattle **(A–E)** and Mediterranean buffalo (animals **F–K**). Large symbols indicate the weighted center of all samples from a group. The diets containing maize grains grown on soil that was treated (Diet M) or not (Diet C) with a commercial AMF preparation are also indicated. Gray dotted arrows indicate the direction of the cross-over from the first to the second experimental period. Black solid arrows depict the 11 ASV's whose abundance most accurately fits the shown variation. The longer the arrow. the stronger the association with the samples in that direction. Due to space restrictions on the plot these arrows are numbered and the corresponding ASV identification number and taxonomic annotation is stated in this legend. Annotation is only given to the taxonomic level [i.e., phylum (p), class (c), order (o), family (f), or genus (g)] to which it can be reliably annotated. The ASV details are: [1] ASV 350279275 = f_Prevotellaceae.g_; [2] ASV 35027951 = g_Prevotellaceae_UCG-001; [3] ASV 35027962 = g_Prevotellaceae_NK3B31_group; [4] ASV 350279171 = g_Prevotella_1; [5] ASV 3502796 = g_Prevotella_1; [6] ASV 35027983 =p_Bacteroidetes.f_F082.g_; [7] ASV 3502793 = p_Bacteroidetes.f_F082.g_; [8] ASV 35027920 = p_Bacteroidetes.f_p-251-o5. g_uncultured _bacterium; [10] ASV 35027993 = g_Christensenellaceae_R-7_group; [11] ASV 350279543 = g_Lachnospiraceae_XPB1014_group.

= 0.9), host type significantly affected rumen prokaryotic community composition and explained 9.3% of the total microbiota variation (P <0.001). The difference in host type can be clearly observed in the RDA biplot (**Figure 2**). Of the 11 plotted ASVs, of which the variation in relative abundance was most accurately fitted with the model, two (numbered 8 and 11 in **Figure 2**) were positively associated with buffalo. Four of the 11 plotted ASVs were positively associated with cattle (numbered 5, 6, 7, and 9 in **Figure 2**). Subsequently, an unpaired Wilcoxon test was used to identify ASVs the relative abundance of which was significantly different between host types. Nine of the 960 ASVs (i.e., 0.94%) were significantly affected by host type (**Table 3**). Four of the nine had a higher mean relative abundance in buffalo compared to cattle, and these belonged to the genera *Butyrivibrio*, *Prevotella* 1, *Methanobrevibacter* and the Bacteroidetes unclassified family F082. The other five had a lower mean relative abundance in buffalo compared to cattle, and these all belonged to the Bacteroidetes phylum: two ASVs belonged to *Prevotella* 1 and the others belonged to the families Muribaculaceae, p-251-o5 and F082.

As well as host type, experimental period also had a significant (P = 0.012) but small affect on the prokaryotic community compostion, explaining 3.1% of the total microbiota variation. The effect of the experimental period is evident from the uniform direction of the arrows which depict time (**Figure 2**). Independent of the dietary intervention, samples from the first and second experimental period are located toward the bottom and top half of the plot, respectively.

DISCUSSION

Based on current knowledge, it was hypothesized that maize grain grown on soil inoculated with AMF would affect ruminal fermentation and associated microbiota, and that this effect would differ between buffalo and cattle. This study clearly showed that presence of AMF during production of maize grains had no effect on either host type in terms of ruminal fermentation parameters, microbial concentrations or prokaryotic community composition. This is in contrast to findings of our previous study, with lactating dairy cows, where rumen bacterial and protozoal concentrations were found to increase (32). However, the findings of the current study is perhaps not surprising considering the limited differences that were observed in the chemical composition of diets M and C (**Table 1**). The lack of effect of the commercial AMF soil treatment on the resulting ruminal utilization of the maize grains indicates

TABLE 3 | The effect of host type on ASVs was compared using an unpaired Wilcoxon test with data normalized using relative abundance and P values adjusted using the Benjamini–Hochberg procedure to decrease the false discovery rate.

ASV identification number	Assigned Taxonomy	% mean change in relative abundance in Buffalo compared to Cattle*	Adj. *P*-value
350279105	p__Firmicutes.f__Lachnospiraceae.g__*Butyrivibrio*_2	0.31	0.006
35027920	p__Bacteroidetes.f__p-251-o5.g__uncultured_bacterium	−0.97	0.009
35027983	p__Bacteroidetes.f__F082.g__	−0.35	0.015
350279131	p__Bacteroidetes.f__Prevotellaceae.g__*Prevotella*_1	0.20	0.034
35027944	p__Bacteroidetes.f__Prevotellaceae.g__*Prevotella*_1	−0.34	0.036
35027973	p__Bacteroidetes.f__Prevotellaceae.g__*Prevotella*_1	−0.54	0.036
35027977	p__Bacteroidetes.f__Muribaculaceae.g__uncultured_rumen_bacterium	−0.40	0.036
350279541	p__Euryarchaeota.f__Methanobacteriaceae.g__*Methanobrevibacter*	0.23	0.036
350279322	p__Bacteroidetes.f__F082.g__	0.20	0.036

**A positive value indicates that the relative abundance of the ASV was higher in buffalo and a negative ASV indicates that it was higher in cattle.*

there are no issues associated with the use of these grains in ruminant feed.

The maize grain, however, is only one part of the maize crop. Maize silage for ruminants is normally prepared using the whole plant. However, AMF inoculation has been reported not to affect subsequent maize silage composition, and when fed to dairy cows also had no impact on milk yield (28). Similarly, no impact of grain produced on soil inoculated with AMF on milk yield was found in our previous study, although a beneficial increase in milk protein yield did occur (32). This would suggest that irrespective of which part of the plant is used as a ruminant feedstuff, AMF inoculation of maize crops has no subsequent deterimental impact on ruminal processes or ruminant productivity. However, as in this study the commercial AMF preparation used also contained some other biostimulating microbes, comparisons between studies that use commercial products containing AMF and those that use only AMF should be made cautiously.

Host type affected protozoal concentration as well as prokaryotic community composition. Differences between cattle and buffalo have been previously reported in terms of ruminal fermentation, microbial counts, rumen degradability, and digestibility of CP and hemicellulose (3, 5, 7, 8, 46). Longer retention time of feed particles in the rumen has also been reported for buffalo bulls compared to Friesian bulls (4). As ruminal feed retention time of feed influences its ruminal digestion (due to having more time to be degraded by the rumen microbiota), buffaloes are likely to degrade nutrients more extensively than cattle (5). Whilst DMI was not measured in this study, it is likely that differences in DMI reported previously (32) contributed to the higher VFA concentration of buffaloes. However, differences in feed intake pattern between the host types over the day may also be a contributing factor.

It is also important to consider that in this trial the cattle's diet was used for the buffalo, which is richer in nutrients relative to the buffalo's dietary requirements (see **Supplementary Table 1**). The significantly higher ruminal total VFA concentration in buffalo, compared to cattle, is similar to other data reported by Parmar et al. (47) where buffalo were also given a high concentrate diet. However, despite the higher VFA concentration in buffalo, we did not observe an associated decrease in pH. Buffalo fed the same basal diet on the research farm where this study was conducted usually have a ruminal pH between 6.7 and 7. This pH is higher than was observed in this study. As such, there may well have been a reduction in the buffalo rumen pH during the study due to the high concentration of VFA.

Buffaloes were found in this study to have a higher lactate concentration and molar proportion of isobutyrate. The 3-fold higher concentration of lactate in buffalo compared to cattle in this study is likely to be due to the more efficient ruminal feed degradation in buffalo. Wanapat and Pimpa (48) reported that lactate in the rumen liquid increased significantly with the increase in the dietary proportion of concentrate. According to Nikolov (49), high lactate concentration in the rumen can occur when it is not completely metabolized to propionate. The animal attempts to neutralize it by increasing buffering capability by enhancing salivation. This is normally also associated with increasing amounts of ammonia in the rumen, as was also observed in this study. The increased lactate concentration was not associated with a decreased pH relative to the cattle fed the same diet, and there was no evidence of the buffalo suffering from subacute ruminal acidosis (49).

Branched-chain VFAs such as isobutyrate are required by many rumen bacteria for protein synthesis, particularly cellulolytic bacteria (50). Isobutyrate supplementation of cattle diets has been reported to improve nutrient utilization and ruminal fermentation characteristics (51). As such, it is speculated that the higher molar proportion of isobutyrate in buffalo, compared to cattle, positively affects their ruminal degradation of feed.

The finding of a significantly lower butyrate molar proportion in buffalo, compared to cattle, was consistent with a previous study that compared swamp buffalo with beef cattle (7). Furthermore, it has also been reported that water buffalo have a lower ruminal butyrate concentration relative to Jersey cows (9). The reason for the increased butyrate molar proportion in cattle

in this study may be due to it being produced by one or more of the bacteria represented by the five ASVs that were significantly increased in cattle compared to buffalo. However, in this study this cannot be confirmed as the Bacteroidetes F082 and p-251-o5 families are uncultured, fermentation products of members of the Muribaculaceae family have not yet been described (52) and ruminal *Prevotella* spp. do not produce butyrate (53).

It is interesting to note that one of the ASVs annotated as the butyrate producing genus *Butyrivibrio* 2 was found to be significantly decreased in cattle compared to buffalo. This is the opposite from what would be expected based on the butyrate molar proportion data. However, due to the higher total VFA in buffalo, there is actually more butyrate production in buffalo compared to cattle.

Three other ASVs belonging to the genera *Prevotella* 1, *Methanobrevibacter,* and the Bacteroidetes unclassified family F082 were also significantly increased in buffalo compared to cattle. ASVs from *Prevotella* 1 and the Bacteroidetes F082 family were also significantly increased in cattle. This indicates that within this genus and uncultured family significant ecological diversity exists with respect to bovine host type. *Prevotella* spp. are known to be involved in protein degradation and polysaccharide digestion (54), however, the ruminal role of the Bacteroidetes unclassified family F082 is not yet known. In contrast, the role of hydrogenotrophic *Methanobrevibacter* in the rumen is well known due to it's central role in ruminal methane production. An increase in *Methanobrevibacter* in buffalo compared to cattle is consistent with the higher VFA production in these animals, which will also result in more hydrogen being available for methane production. Predictive equations have been used to estimate the enteric methane production in buffalo, however, this needs to be directly assessed *in vivo* (55, 56).

In this study, a higher protozoal concentration was found in buffalo, compared to cattle. This result is consistent with findings of other studies (9, 57). As methanogenic archaea are extra- and intra-cellulary associated with protozoa, this may also explain the higher relative abundance of *Methanobrevibacter* in buffalo compared to cattle. Although the protozoal community composition was not analyzed in the present study, it has been previously shown to differ between water buffalo and Jersey cows (9). In sheep, increased protozoal concentrations have been associated with a decreased rumen bacterial concentration (due to their bacteriolytic activity) and increased ammonia and VFA concentrations (58). However, in our study no differences between buffalo and cattle were found in terms of ammonia or bacterial concentrations. It is now realized though that the role of protozoa in the rumen is more complex than previously thought, as indicated by a review which included a meta-analysis of over 20 different defaunation studies (59).

As well as host type effects on the rumen prokaryotic composition, an effect of experimental period was evident. The reason for this is not clear, and may be related to environmental factors. Temperature could be an indirect trigger to shift rumen community composition, via changes in the physiology of animals in response to heat stress (60, 61). For example, the animal housing area was not temperature controlled and the ambient temperature in the first experimental period (23.9 + 1.7°C) was lower than that of the second experimental period (27.6 + 1.0°C). Regardless though, this finding confirms the importance of using a cross-over design when comparing different diets within the same animals.

CONCLUSION

Based on current knowledge, it was hypothesized that maize grain produced in the presence of AMF would affect ruminal fermentation and associated microbiota, and that this effect would differ between buffalo and cattle. This was proven not to be the case as no effect on ruminal fermentation or the associated ruminal microbiota in either cattle or buffaloes was observed when feeding maize grains produced from crops grown on soil treated with a commercial AMF preparation. Considering the beneficial effect of AMF on maize cultivation, in terms of environmental and economic sustainability of forage production, this is clearly a positive outcome. Prokaryotic community composition, protozoal concentrations, and several ruminal fermentation parameters significantly differed between buffalo and cattle, even though they were fed and managed under the same conditions. The different nutrional requirements of these non-lactating animals may have been a contributing factor to this, along with previously reported differences in digestive physiology and rumen retention time between these two bovine species.

DATA AVAILABILITY STATEMENT

The raw sequence data generated in this study is deposited in ENA under study accession number PRJEB37928, with individual sample accession numbers ranging from ERS4525212-ERS4525234. **Supplementary Material** includes supplementary methods (diet composition, feed chemical analysis, rumen fermentation analysis, and DNA extraction), host nutritional requirements (**Supplementary Tables 1**, **2**), phylum level prokaryotic community composition data for individual animals (**Supplementary Figure 1**), genus level prokaryotic community composition data for individual animals (**Supplementary Figure 2**), and weighted and unweighted UniFrac NMDS plots (**Supplementary Figure 3**).

ETHICS STATEMENT

The animal study research protocol was approved by the National Ethics Committee (Ministry of Health Decree 26/2014, authorization n°399/2018, Italy) in accordance with the guidelines established by the EU Council/Directives 86/609/EEC.

AUTHOR CONTRIBUTIONS

AC designed and performed the experiment. DM and SD performed feed analyses and processing. GC performed the statistical analysis and interpretation of univariate data. AC together with JE performed the rumen prokaryotic community composition and microbial concentration analysis.

GH performed the statistical analysis of the rumen prokaryotic community composition data. AC, JE, and GH wrote the manuscript with input from all authors. All authors approved the final manuscript.

FUNDING

This study was part of a larger project (Use of non-toxigenic strains of *Aspergillus flavus* and mycorrhized microbial consortia for the reduction of the mycotoxin content of maize), which was funded by the FEASR, Rural Development Program 2014–2020, Lombardy Region, Italy (N° 9571 30/9/2016). AC also gratefully acknowledges CREA for funding her short stage placement at WUR, during which the rumen prokaryotic community composition and microbial concentration analysis was performed.

ACKNOWLEDGMENTS

Assistance provided during sampling and protozoa counts by Emanuela Rossi was greatly appreciated. We are also grateful to farm workers for animal care.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.556764/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 16 October 2020
doi: 10.3389/fvets.2020.574434

Assessment of Multicolor Flow Cytometry Panels to Study Leukocyte Subset Alterations in Water Buffalo (*Bubalus bubalis*) During BVDV Acute Infection

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 19 June 2020
Accepted: 04 September 2020
Published: 16 October 2020

Citation:
Grandoni F, Martucciello A, Petrini S, Steri R, Donniacuo A, Casciari C, Scatà MC, Grassi C, Vecchio D, Feliziani F, De Matteis G, Davis WC and De Carlo E (2020) Assessment of Multicolor Flow Cytometry Panels to Study Leukocyte Subset Alterations in Water Buffalo (Bubalus bubalis) During BVDV Acute Infection. Front. Vet. Sci. 7:574434. doi: 10.3389/fvets.2020.574434

The identification of cross-reactive monoclonal antibodies (mAbs) that recognize orthologous leukocyte differentiation molecules (LDM) in buffaloes has overcome a major impediment limiting research on the immune response to pathogens and development of vaccines. As reported, two pilot trials were conducted to accomplish two objectives: (1) demonstrate that multiparameter flow cytometry can be conducted equally well in buffalo with mAbs directly and indirectly labeled with fluorochromes in research and (2) flow cytometry can be used to compare and extend studies on diseases of economic importance to buffalo using bovine viral diarrhea virus (BVDV) as a model pathogen. Pregnant buffalo cows were infected with BVDV-1 at 81 (trial 1) and 203 (trial 2) days post artificial insemination and flow cytometric evaluations were performed at 0, 3, 4, and 14 days after infection (dpi). Fluorochrome conjugated mAbs were used in trial 1, and fluorochrome conjugated goat isotype specific anti-mouse antibodies were used to label mAbs in trial 2. Flow cytometric analysis revealed a transient lymphopenia occurs during the 1st days following infection similar to lymphopenia reported in cattle. In particular, significant differences were observed between pre- and post-infection absolute values of T lymphocytes (−56%, $P < 0.01$). $CD21^+$ B lymphocytes (−65%, $P = 0.04$), and Natural Killer cells (−72%, $P < 0.001$). No significant differences were observed in monocytes and neutrophil absolute values, or the CD4:CD8 ratio. Animal health status was followed until 15 days after calving. No clinical signs of infection were observed during the evaluation period, however, animals in trial 1 developed complications later the infection. One cow aborted at 57 days post-infection, the second cow developed a prolapse a day after calving and died. These two animals

also showed a more pronounced lymphopenia in comparison with animals infected at 203 days of pregnancy (e.g., −77 vs. −22% T lymphocytes at 3 dpi, respectively). The pilot studies have demonstrated that it is possible to use multicolour multiparameter flow cytometry to study the immune response to pathogens affecting the health of buffalo.

Keywords: flow cytometry, monoclonal antibody, water buffalo (*Bubalus bubalis*), bovine viral diarrhoea virus (BVDV), experimental infection

INTRODUCTION

Flow cytometry (FCM) is an important and versatile technology: flexibility, accuracy and multiparametric analysis are some of its characteristics. It has proven useful in research in microbiology, oncology, immunology, hematology, and nanotechnology. Flow cytometry was introduced into veterinary sciences in the 80's concurrent with pioneering studies to develop monoclonal antibodies (mAbs) against leukocyte differentiation molecules (LDM) in cattle and other livestock species (1–3). During this same timeframe studies were initiated to make use of the first characterized mAbs to determine the frequency of leukocyte subsets in blood and milk (4, 5). Studies were also initiated to determine if flow cytometry could be used to study sequential changes in the frequency of leukocyte subsets associated with pathogenesis of livestock pathogens starting with a persistent pathogen, BVDV in cattle (6). Excluding cattle, the use of flow cytometry in studies with other veterinary species has been constrained, attributed to the limited availability and cost of developing mAb reagents for research (7). This has included research with water buffalo. Although buffaloes are an important livestock species, to date, no resources have been available to develop mAbs against buffalo leukocyte differentiation molecules (LDM) for use in the study of the immunopathogenesis of pathogens and development of vaccines. Fortunately, it has been possible to take advantage of the phylogenetic relation of buffalo with cattle to circumvent this constraint. The antigenic composition of many of the molecules expressed on leukocytes have been conserved. Screening of mAbs developed against LDM in cattle, sheep, and goat revealed a large set of the mAbs recognize epitopes conserved on orthologous LDM in buffalo (8–11). The set of cross-reactive mAbs now make it possible to conduct studies on the immune response to pathogens equivalent to ongoing studies in cattle. The present study was undertaken to introduce the use of the mAbs in flow cytometry to conduct studies on the immunopathogenesis of pathogens in buffalo, using BVDV as a model of a pathogen with respect to which further research is needed to fully understand the mechanisms of pathogenesis. BVDV is a member of the *Flaviviridae* family, genus *Pestivirus* (12). The International Committee on Taxonomy of Viruses (ICTV) recognizes two established species namely bovine viral diarrhea virus type 1 (BVDV-1) and type 2 (BVDV-2), and a third putative bovine species, referred to as HoBi-like pestivirus (13). This is one of the groups of viral pathogens affecting livestock. Studies in cattle have revealed exposure usually leads to development of mild disease characterized by pyrexia, anorexia, leukopenia, and mild diarrhea. However, infection can also lead to development of mucosal disease and reproductive problems (abortion, teratogenesis, embryo resorption, fetal mummification, and stillbirth). Cows infected before 150 days of gestation can give birth to calves persistently infected with BVDV (12). The mechanism of bypassing the adaptive immune system by establishing tolerance permits the virus to be extremely successful by used an evasion strategy that still remains to be fully determined. A main problem with BVDV is that infection leads to development of an immune response that controls but does not clear infection. In spite of the presence of antibody and cell mediated immunity, the virus continues to persist and replicate in infected animals and is released into the environment. Infected cows and calves serve as reservoirs contaminating the environment. Most of the reported BVDV infections refer to infections in the *Bovidae* family. Only a few of them refer to infection in buffaloes, and most of these have been reports on seroprevalence (14–16). The presence of the BVDV-1 RNA has been demonstrated in fetal serum in buffaloes with mucosal disease in Australia (17) and Argentina (18). In Italy, viruses of sub-genotype BVDV-1a (19) and BVDV-1b (20) have been isolated from aborted fetuses. Important to expanding opportunities to conduct research in buffalo, information was obtained in the present study showing flow cytometry can be used with mAbs directly and indirectly labeled with fluorochromes. In addition data were obtained showing infection with BVDV leads to transient leukopenia similar to infection in cattle and fetal development. The availability of the large set of cross-reactivity mAbs now affords opportunity to expand research on the immune response to pathogens and vaccine development in buffalo.

MATERIALS AND METHODS

Animals and Experimental Design

The experimental protocol for the study was authorized by the Ministry of Health (authorization n. 503/2017-PR) on the basis of Legislative Decree 26/03/14 on the protection of animals used for scientific purpose. The authorization limited the study to use of four healthy Mediterranean buffalo cows (6 years of age), negative for BVDV antibodies and antigens. Two cows were infected at 81 days (trial 1), the second two cows were infected at 203 days (trial 2) after artificial insemination. The pregnancy status was determined by clinical examination. During the infection trials, the animal health status was observed daily for a month after infection and then every 3 days until 15 days after calving.

Virus

A non-cytopathic (ncp) field strain of BVDV-1 was used for experimental infection. Virus used in the study were prepared

from the second passage in bovine kidney cells (MDBK). All animals were infected with a field type of BVDV-1. The virus was given by intranasal route at a dose of 2.5 mL × $10^{5.00}$ $TCID_{50}$/mL for each animal.

Sample Collection

Peripheral blood was collected from jugular vein into vacutainer tubes containing K_3-EDTA, Li-Heparin and anticoagulant-free tubes were used for hematological, flow cytometric and serological analyses, respectively (Becton Dickinson, Plymouth, UK). Samples were collected at: 0, 3, 4, 6, 8, 10, 14, 17, 20, and 27 days post-infection (dpi). Nasal and vaginal swabs were taken at after day 10 dpi. Blood and ear notch samples were collected from calves immediately after birth and tested for presence of BVDV antigen and antibody.

Hematological, Serological, and Molecular Analyses

Total and differential leukocyte counts were performed with a Cell Dyn 3700 (Abbott, Abbott Park, IL, USA) according to standard operation procedures. All serum samples were tested for BVDV antibodies and antigen by the commercial ELISAs ID Screen® BVD p80 Antibody Competition (IDvet, Grabels, France) and Bovine Viral Diarrhea Virus BVDV antigen test kit/serum plus (IDEXX, Westbrook, ME, USA), following the manufacturer's instructions. EDTA blood samples, nasal and vaginal swabs and ear notch samples were used for molecular analysis. Total RNA was extracted using the QIAampViral RNA mini Kit (Thermo Fisher Scientific, Waltham, MA, USA) as instructed by the manufacturer. The tests performed were an end point PCR that amplifies the 5'-UTR region using the pair of 324/326 primers amplifying a fragment of 288 bp (21) and the real-time RT-PCR described in the OIE Manual (22).

Flow Cytometry: Monoclonal Antibodies and Labeling of Cells

The mAbs used in the present study are listed in **Table 1**. In a pre-trial study, we assessed the cell viability by LIVE/DEAD Fixable Near-IR stain kit (Thermo Fisher Scientific, Waltham, MA, USA). Since the viable cells were always higher than 95%, live/dead staining was omitted in trials 1 and 2. In the same study we compared the labeling efficiency of anti-human and anti-bovine mAbs cross-reactive with buffalo river type LMD orthologs (9, 10) including anti-bovine CD4 clone CC8 cross-reactive with buffalo swamp type (23). Flow cytometric analyses were performed: in the first trial at 0, 3, 4, and 14 dpi in the second trial at 0, 3, 4, 6, 8, 10, 14, and 17 dpi. In trial 1 we used a direct labeling method with purified mAbs labeled in house: clone IL-A11a using Zenon™ R-Phycoerythrin Mouse (Thermo Fisher Scientific, Waltham, MA, USA), MM1a and GB21a clones using Lightning-Link® APC (Allophycocyanin) and PE-Cy7 Conjugation Kit (Abcam, Cambridge, UK) respectively, following the manufacturer's instructions.

Fifty microliters of whole heparinized blood sample were incubated with saturating concentrations of mAbs in a final volume of 100 μL with phosphate buffered saline (PBS, pH 7.2) for 15 min at 4°C in the dark. Following incubation, erythrocytes were lysed with 1.0 mL of Tris-buffered ammonium chloride solution (0.87% w/v, pH 7.3) for 10 min, then mixed with 2 mL of cold PBS and then centrifuged at 300 g for 5 min. Pellets of labeled cells were resuspended in 150 μL of cold PBS for flow cytometric analysis. In trial 2 we used mAbs indirectly labeled with fluorochrome conjugated goat isotype specific anti-mouse antibodies. In brief, 2.0 mL of anticoagulated whole blood was added to a 50 mL polypropylene tube and lysed with 20.0 mL of the same ammonium chloride solution. The cells were sedimented by centrifugation and then resuspended in 25 mL of cold PBS. 5 × 10^5 cells were incubated with 300 ng of each mAb in a final volume of 100 μL of PBS for 15 min at 4°C in the dark. Cells were washed (300 g for 5 min) then incubated under the same conditions using goat anti-mouse isotype specific secondary antibodies at optimal dilutions. Following incubation for 15 min, the cells were washed in PBS. The cells were resuspended in 150 μL for flow cytometric analysis. Fluorescence Minus One (FMO) controls were used to the correct quadrant positions for CD8, CD14, CD21, CD25, and CD209 molecules. A CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA) was used to collect at least 5 × 10^4 leukocytes. Data were analyzed using Kaluza software v. 2.1 (Beckman Coulter, Brea, CA, USA). For each lymphocyte subset, we applied the specific gating strategy (**Figure 1**), calculating the percentage of the subsets on total lymphocytes obtained in the forward scatter (FSC) *vs.* side scatter (SSC) dot plot. The relative percentages were then used to estimate the absolute value of the subset using the absolute values produced by Cell Dyn.

Statistical Analyses

For each hematological and cytometric variable, variation time dependent was investigated by fitting the following mixed linear model with the MIXED procedure of SAS/STAT 9.2 (SAS Institute Inc., Cary, NC, USA) (24):

$$Y = \mu + TRIAL + DPI + TRIAL^{*}DPI + c + e$$

where y = hematological or cytometric parameters; μ =overall mean; TRIAL = fixed effect of the infection (two classes: 1, 2); DPI = fixed effect of the days post infection (10 classes: 0, 3, 4, 6, 8, 10, 14, 17, 20, and 27 for hematological and four classes: 0, 3, 4, and 14 for cytometric parameters respectively); c = random effects of individual buffalo cow; e = random residual.

RESULTS

Clinical Observations

The experimentally infected animals showed no change in rectal temperature, signs or clinical symptoms. Later, however, trial 1 animals presented severe clinical complications: Buffalo-1 aborted 57 days after infection (138 days of gestation), while Buffalo-2 presented prolapse the day after calving and died.

Serological and Molecular Results

All animals showed seroconversion, between 14 and 27 dpi, and all calves born from these buffaloes had BVDV antibodies and were negative for viral antigen, except the calf born from Buffalo-2 (trial 1) which was negative for both. Except for a trial 2 animal,

TABLE 1 | Multicolor flow cytometric combinations used in this study.

Panel-method	Antigen	Clone	Isotype	Source	Labeling	References
1-indirect	CD8	7C2B	IgG_{2a}	WSU-MAC	Fitc	(10)
	CD4	GC50A	IgM	WSU-MAC	PE	(10)
	δ chain	GB21A	IgG_{2b}	WSU-MAC	PE-Cy7	(10)
	CD3	MM1a	IgG_1	WSU-MAC	APC	(10)
2-indirect	CD25	LCTB2A	IgG_3	WSU-MAC	Fitc	(10)
	ACT16	GB110a	IgM	WSU-MAC	PE	(10)
	CD4	IL-A11a	IgG_{2a}	WSU-MAC	PE-Cy7	(10)
	CD3	MM1a	IgG_1	WSU-MAC	APC	(10)
3-indirect	CD25	LCTB2A	IgG_3	WSU-MAC	Fitc	(10)
	ACT16	GB110a	IgM	WSU-MAC	PE	(10)
	CD8	7C2B	IgG_{2a}	WSU-MAC	PE-Cy7	(10)
	CD3	MM1a	IgG_1	WSU-MAC	APC	(10)
4-indirect	CD25	LCTB2A	IgG_3	WSU-MAC	Fitc	(10)
	δ chain	GB21A	IgG_{2b}	WSU-MAC	PE	(10)
	CD8	7C2B	IgG_{2a}	WSU-MAC	PE-Cy7	(10)
	CD335	AKS1	IgG_1	Bio-Rad	APC	(10)
5-indirect	CD25	LCTB2A	IgG_3	WSU-MAC	Fitc	(10)
	CD209	209MD26A	IgG_{2a}	WSU-MAC	PE	(10)
	CD14	CAM66A	IgM	WSU-MAC	PE-Cy7	(10)
	CD21	GB25A	IgG_1	WSU-MAC	APC	(10)
6-direct	CD8	CC63	IgG_{2a}	Bio-Rad	Fitc	(9)
	CD21	CC21	IgG_1	Bio-Rad	Fitc	(9)
	CD4	IL-A11a	IgG_{2a}	WSU-MAC	PE	(10)
	CD335	AKS1	IgG_1	Bio-Rad	PE	(10)
	CD14	TÜK4	IgG_{2a}	Bio-Rad	PE-Cy5.5	(9)
	WC1-N2	BAQ4A	IgG_1	WSU-MAC	PE-Cy7	(10)
	CD3	MM1a	IgG_1	WSU-MAC	APC	(10)

WSU-MAC = Washington State University-Monoclonal Antibody Center (Pullman. WA, USA); Bio-Rad Laboratories (Hercules, CA, USA). The anti-bovine CD4-PE conjugated (clone CC8, Bio-Rad), since negative, was not included in panel 6.

PCR was positive at 3, 6 (nasal and vaginal swabs), and 8 dpi (nasal swap), blood samples, nasal, and vaginal swabs from other animals were negative for viral RNA.

Hematological Analyses

As summarized in **Table 2** and in **Supplementary Figure 1**, the experimental infection caused marked leukopenia due to a statistically significant lymphopenia. All animals showed the same trend: a rapid diminution in lymphocytes first evident at 3 dpi, with low values up to 8 dpi, then an increase at 10 dpi. A subsequent decrease occurred from 14 to 27 dpi, although the differences were not statistically significant. The other leukocyte subpopulations did not show significant changes or evident trends post-infection due to a marked variability between animals. In trial 1, the infection effects were more evident. Lymphopenia reached higher levels (−66 vs. −50%) than trial 2 (**Supplementary Table 1**), and the increase shown at 10 dpi respect to 0 dpi was more pronounced in the second than first infection trial (+78 vs. −1%).

Antibody Labeling Evaluation by Flow Cytometry

All monoclonal antibodies used in trials 1 and 2 (**Table 1**) showed comparable labeling efficiency evaluated by percentage of positive cells. Unexpectedly the CC8 clone anti-bovine CD4 was negative, although it had been reported as positive swamp water buffaloes (23).

Flow Cytometric Evaluation of Lymphocyte Subset Alterations

Statistical assessments were done using time points 0, 3, 4, 14 dpi common to both trials. The absolute values and percentage of differences between pre- and post-infection values are summarized in **Table 3** and **supplementary Table 1**. Since the pre-infection absolute values of lymphocyte subsets, excluding B cells, obtained by direct (trial 1) or indirect labeling (trial 2) methods were similar, we considered the two flow cytometric approaches comparable. On average, the two infections produced severe lymphopenia due to the general decrease of all lymphocyte subsets (**Table 3**). T lymphocytes ($CD3^+$) decreased significantly

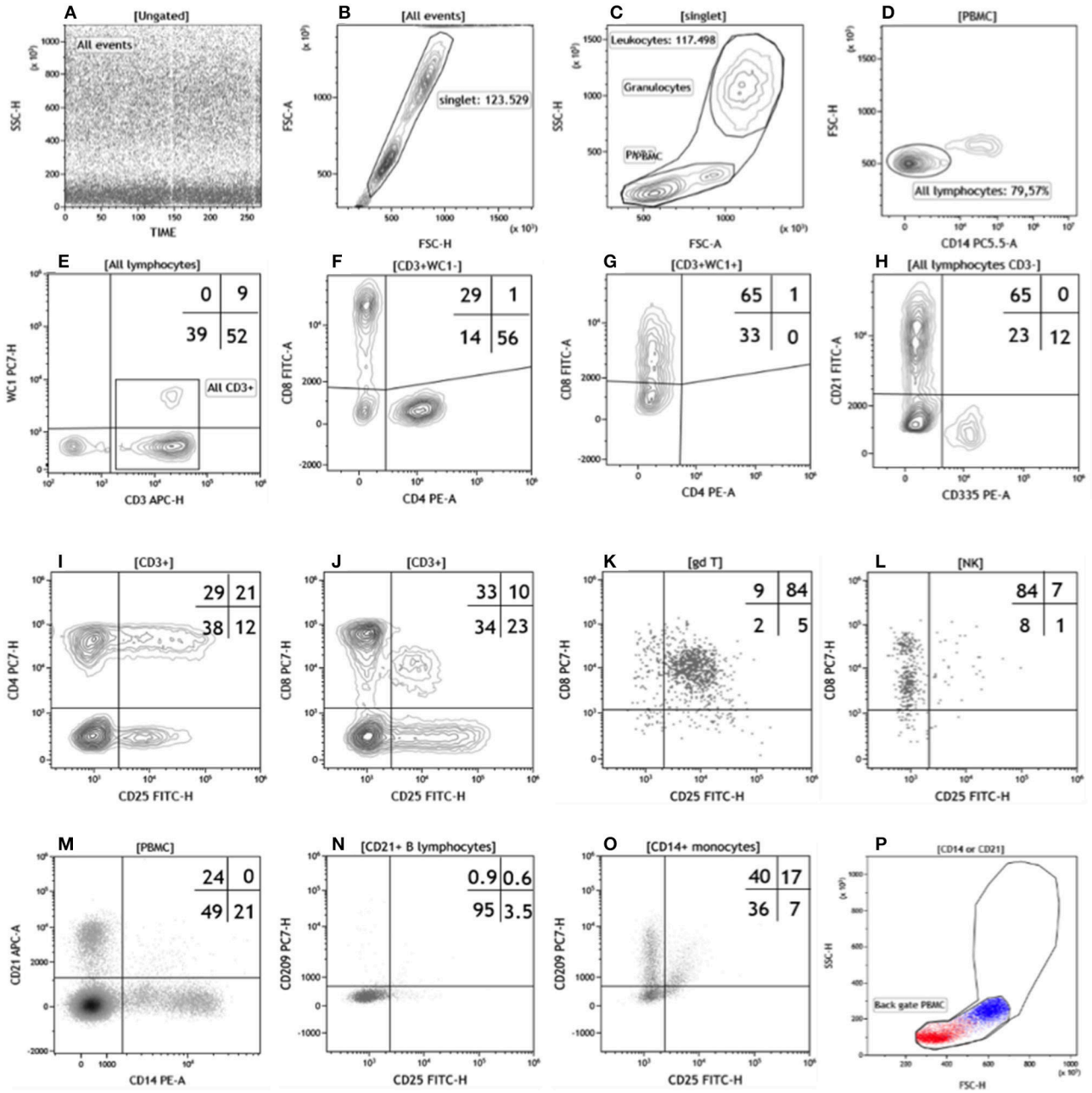

FIGURE 1 | Flow cytometric gating strategy used in this study. The gating strategy **(A–H)** used to identify the lymphocyte subsets in Trial 1 was: **(A)** time parameter vs. SSC or FL to exclude event burst, **(B)** FSC-H vs. FSC-A to exclude doublets, **(C)** FSC vs. SSC to exclude debris, damaged and/or dead cells, then identify total leukocytes and PBMC, **(D)** CD14 vs. WC1 to identify total T lymphocytes and the WC1 subset. To characterize the other T lymphocyte subsets we used CD4 vs. CD8 for both $WC1^{-}CD3^{+}$ **(F)** and $WC1^{+}CD3^{+}$ **(G)** T cells. In buffalo the CD8 antigen is expressed by most γδ T lymphocytes (10), then we calculated the absolute value of $CD3^{+}CD8^{+}$ (**Table 3** and **Supplementary Table 1**) as the total of upper left quadrant events in **(F,G)** dot plots. All lymphocytes $CD3^{-}$ (bottom left in the dot plot **E**) were used to identify $CD21^{+}$ B lymphocytes (top left) and NK cells ($CD335^{+}$ bottom right) in the **(H)** dot plot. The gating strategy **(I–O)** used in Trail 2 had in common the first three gates (not shown) of Trail 1 **(A–C)**. For Panel 1, a CD3 vs. δ chain CD4 or CD8 dot plots were used to identify T lymphocyte subsets (not shown). In the Panel 2 and 3 we used a CD3 vs. SSC dot plot to identify all CD3 positive lymphocytes while CD3 vs. CD4 or CD8 dot plots to identify CD4 or CD8 T subsets, respectively (not shown). In the dot plot CD25 vs. CD4 **(I)** or CD8 **(J)**, the $CD3^{+}$ gate was used to evaluate the percentage of CD4 or CD8 expressing CD25. In Panel 4, the δ chain vs CD335 dot plot was used to identify γδ T lymphocytes and NK cells (not shown) and then differentiated in the CD25 vs. CD8 dot plot for the co-expression of the two antigens (**K,L**, respectively). In Panel 5, the PBMC gate was used to identify $CD21^{+}$ B lymphocytes and $CD14^{+}$ monocytes **(M)**, which were

(Continued)

FIGURE 1 | subsequently characterized by co-expression of CD25 and CD209 antigens (**N,O**, respectively). Finally back gating on dot plot FSC vs. SSC confirmed the validity of the gating strategy **(P)**. For a better separation of cell clusters, we used the most appropriate combinations of fluorescent parameters (Hight-H and Area-A). For a better visualization we show the results as density dot plot **(A,K–O)**, contour plots **(B–J)**, and color dot plot **(P)** with a blue ($CD14^+$ monocytes) and red ($CD21^+$ B lymphocytes) color coding.

TABLE 2 | Comparison of total and differential leukocyte absolute counts (10^9 cells/L) of infected buffaloes.

	dpi	WBC		Neutrophils		Lymphocytes		Monocytes		Eosinophils		Basophils	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Trial 1 ($N = 2$)	0	6.29	0.33	2.66	0.42	2.87	0.59	0.55	0.09	0.17	0.07	0.04	0.00
	3	5.15	0.21	3.11	0.65	1.08	0.58	0.65	0.01	0.28	0.16	0.03	0.02
	4	3.54	0.62	1.83	0.54	1.00	0.19	0.48	0.05	0.17	0.24	0.06	0.07
	6	4.16	1.05	2.17	0.87	0.97	0.12	0.89	0.10	0.10	0.02	0.03	0.02
	8	4.80	0.51	2.45	0.02	1.35	0.62	0.90	0.19	0.01	0.00	0.10	0.05
	10	6.31	0.93	2.46	0.79	2.83	0.22	0.70	0.26	0.29	0.07	0.02	0.02
	14	6.34	0.31	3.23	0.76	2.06	0.11	0.84	0.31	0.19	0.02	0.03	0.02
	17	7.94	1.39	4.78	0.72	2.34	0.54	0.65	0.13	0.01	0.00	0.17	0.01
	20	6.66	1.23	3.12	0.54	2.58	0.64	0.69	0.13	0.23	0.12	0.05	0.02
	27	6.77	0.88	3.03	0.06	2.91	1.02	0.45	0.03	0.34	0.04	0.03	0.00
Trial 2 ($N = 2$)	0	7.27	2.43	3.89	1.91	2.16	0.39	0.83	0.20	0.31	0.43	0.09	0.10
	3	5.61	0.59	3.28	0.49	1.38	0.08	0.66	0.29	0.26	0.31	0.06	0.06
	4	5.43	0.44	3.27	0.49	1.12	0.01	0.87	0.03	0.00	0.00	0.17	0.11
	6	5.28	0.05	3.21	0.08	1.15	0.13	0.71	0.15	0.21	0.15	0.04	0.05
	8	5.20	0.16	3.13	0.01	1.08	0.20	0.87	0.03	0.09	0.08	0.05	0.04
	10	8.02	1.61	2.79	0.64	3.85	0.83	0.86	0.09	0.50	0.05	0.01	0.00
	14	6.60	1.73	3.19	1.06	2.30	0.16	0.95	0.49	0.01	0.01	0.15	0.02
	17	6.65	0.54	4.30	0.38	1.57	0.24	0.67	0.17	0.00	0.00	0.09	0.06
	20	7.24	0.98	4.71	0.71	1.81	0.07	0.55	0.16	0.14	0.15	0.04	0.01
	27	6.80	1.77	3.06	0.73	2.58	0.98	0.90	0.09	0.18	0.24	0.09	0.10
Mean infection ($N = 4$)	0	6.78^a	1.53	3.27	1.33	2.51^A	0.58	0.69	0.20	0.24	0.27	0.07	0.06
	3	5.38^a	0.45	3.19	0.48	1.23^B	0.38	0.66	0.17	0.27	0.20	0.04	0.04
	4	4.48^b	1.18	2.55	0.94	1.06^B	0.13	0.68	0.23	0.09	0.17	0.11	0.10
	6	4.72^a	0.89	2.69	0.79	1.06^B	0.14	0.80	0.15	0.16	0.11	0.04	0.03
	8	5.00^a	0.38	2.79	0.40	1.22^B	0.41	0.88	0.11	0.05	0.06	0.07	0.05
	10	7.16^a	1.46	2.63	0.62	3.34^A	0.77	0.78	0.18	0.40	0.13	0.02	0.01
	14	6.47^a	1.03	3.21	0.75	2.18^A	0.18	0.90	0.34	0.10	0.10	0.09	0.07
	17	7.30^a	1.14	4.54	0.55	1.95^A	0.56	0.66	0.12	0.00	0.00	0.13	0.06
	20	6.95^a	0.97	3.91	1.05	2.19^A	0.58	0.62	0.14	0.18	0.12	0.05	0.02
	27	6.78^a	1.14	3.04	0.42	2.74^A	0.84	0.68	0.27	0.26	0.17	0.06	0.07

Significance test was not performed for trials 1 and 2 due to the small number of animals. Significant differences are referred to individual classes of leukocytes (column) and considered between 0 dpi and single time points (e.g., 0 vs. 3 dpi), $^{A,B}P < 0.01$; $^{a,b}P < 0.05$.
WBC, white blood cells (total leukocytes); SD, standard deviation.

by 3 and 4 dpi ($P < 0.01$) with a maximum decrease of −55.8%. At 14 dpi, the absolute values were not statistically different from 0 dpi. $CD4^+$ and $CD8^+$ T cells had a similar dynamic: they decreased by 53.4% ($P = 0.02$) and 56.7% ($P = 0.02$), respectively, and both reached the minimum at 4 dpi. At 14 days while $CD4^+$ T lymphocytes had the same values as the pre-infection, $CD8^+$ T lymphocytes were still −13.6% compared to 0 dpi. The average CD4:CD8 ratio did not change significantly. Statistically significant differences were observed in other cell subsets, $CD21^+$ B lymphocytes ($P = 0.04$) and NK cells ($CD335^+$) ($P < 0.001$). Maximum average decrease (−71.5%) was shown by NK cells at 4 dpi. The absolute number of lymphocyte subsets indicated clearly that the lymphopenia caused by the infection was more pronounced when the animals were infected at 81 days post insemination compared to 203 days of gestation. At 3 dpi, the total $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes decreased by 77.1, 83.8, and 65.4% in trial 1 compared to −22.2, −21.9, and −41.5% in trial 2, respectively. In addition, at 14 dpi, trial 1 animals did not return to their pre-infection values (−30.7, −21.7, and −38.5%) while in trial 2 animals increased by +13.5, +20.4, and

TABLE 3 | Comparison of absolute counts of lymphocyte subsets evaluated by flow cytometry (10^9 cells/L).

		0 Dpi		3 Dpi		4 Dpi		14 Dpi	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Trial 1 (*N* = 2)	$CD3^+$	1.78	0.43	0.41	0.13	0.55	0.07	1.23	0.04
	$CD21^+$ B	0.90	0.13	0.58	0.42	0.32	0.05	0.61	0.10
	NK ($CD335^+$)	0.19	0.03	0.09	0.03	0.05	0.02	0.18	0.00
	$CD3^+WC1^+$	0.26	0.07	0.15	0.06	0.14	0.02	0.15	0.01
	$CD3^+CD4^+$	0.99	0.13	0.16	0.08	0.35	0.11	0.78	0.03
	$CD3^+CD8^+$	0.52	0.20	0.18	0.04	0.19	0.02	0.32	0.03
	CD4:CD8	1.9	0.6	0.9	0.1	2.4	1.2	2.4	0.4
Trial 2 (*N* = 2)	$CD3^+$	1.58	0.16	1.23	0.11	0.93	0.04	1.79	0.34
	$CD21^+$ B	0.41	0.21	0.11	0.03	0.14	0.07	0.37	0.25
	NK ($CD335^+$)	0.17	0.03	0.04	0.00	0.05	0.02	0.13	0.07
	$CD3^+$ γδ T	0.26	0.12	0.15	0.02	0.13	0.01	0.23	0.02
	$CD3^+CD4^+$	0.83	0.17	0.73	0.00	0.55	0.07	1.12	0.22
	$CD3^+CD8^+$	0.57	0.01	0.39	0.08	0.37	0.10	0.70	0.20
	CD4:CD8	1.4	0.3	1.9	0.41	1.5	0.62	1.6	0.15
Mean infection (*N* = 4)	$CD3^+$	1.68^A	0.29	0.82^B	0.48	0.74^B	0.22	1.51^A	0.38
	$CD21^+$ B	0.66^a	0.32	0.34^a	0.36	0.23^b	0.11	0.49^a	0.21
	NK ($CD335^+$)	0.18^A	0.03	0.06^B	0.04	0.05^B	0.02	0.16^A	0.05
	$CD3^+CD4^+$	0.96^{aA}	0.13	0.45^b	0.33	0.45^B	0.14	0.95^a	0.23
	$CD3^+CD8^+$	0.59^{aA}	0.14	0.28^b	0.13	0.26^B	0.14	0.51^a	0.25
	CD4:CD8	1.71	0.50	1.39	0.67	2.05	0.95	2.03	0.53

*Trial 1 values were obtained from multicolor flow cytometry panel 6, trail 2 values from panels 1–5 (**Table 1**). Significance test was not performed for trials 1 and 2 due to the small number of animals. Significant differences are referred to single lymphocytes subset (line) between pre- and post-infection time points: 0 vs. 3 dpi; 0 vs. 4 dpi; 0 vs. 14 dpi.* $^{A,B}P < 0.01$; $^{a,b}P < 0.05$.

+5.8%. The CD4:CD8 ratio showed a clear divergence between the two groups. While in trial 1 the effect of the infection was evident in reduction in the ratio from 1.9 at 0 dpi to 0.9 at 3 dpi (−52.6%), a positive increase to 35.7% was observed in trial 2 (**Table 3**). CD209 expression (trial 2) was always negative on $CD21^+$ B lymphocytes, whereas $CD14^+$ monocytes showed an evident increase from 8 to 17 dpi compared to baseline. At the same time point, we identified a small cellular subset with a phenotype $CD21^-CD14^-CD209^+$ (**Supplementary Figure 2**).

Flow Cytometric Evaluation of Cell Activation Status

In trial 2 we evaluated the cell activation status by CD25 and ACT16 antigen expressions. CD25 (IL-2 receptor) was expressed on 79% of γδ, 38% of $CD4^+$, and 35% of $CD8^+$ T lymphocytes, 23% of $CD14^+$ monocytes, 8% of $CD21^+$ B lymphocytes, and 7% of NK cells. The only difference noted in expression after infection was on all T lymphocytes ($CD3^+$) 8 dpi (+33%). ACT16, a molecule upregulated on *in vitro* activated $CD4^+$ and $CD8^+$ T lymphocytes (10), was never expressed in this pilot trial.

DISCUSSION

Flow cytometry is being used extensively in the study of the immune response to pathogens in humans and some economically important livestock species. A few mAbs specific for bovine LDM, however, are available conjugated to fluorochromes and was therefore useful to identify some cross-reactive anti-human mAbs. Although considerable progress has been made in identifying mAbs for use in buffalo, there is still a need to expand the mAb reagents for use in this species. In the present study we tested the CC8 anti-bovine CD4 mAb that, although cross-reactive with swamp buffalo (23), was negative with river-type buffalo. In cattle we noted that missense mutations Q306H and K310N in exon 5 of CD4 gene prevented the binding of the CC8 mAb (25). In an *in silico* study (unpublished), we verified that the genomic sequences of the river-type buffalo the L-glutamine 306 is conserved, but two Lysines (308 and 310) are changed to Asparagine and Serine, respectively. Although swamp-type buffalo exon 5 sequences are not published, this labeling difference could be an indirect confirmation that the same mutations in river buffalo account for loss of the epitope identified by CC8. In buffalo, moreover, flow cytometric methods are still under development. One of the current issues has been whether there is any difference in the use of mAbs directly or indirectly labeled with fluorochromes. Most of the mAbs for research in livestock are only available unconjugated with fluorochromes. For profit companies have developed three methods for labeling mAbs with fluorochromes: (1) goat fluorochrome polyclonal and isotype specific anti-mouse

antibodies for use in indirectly labeling mAbs; (2) Zenon fluorochrome conjugated isotype specific Fab for binding to mAbs immediately for use in multicolor flow cytometry; (3) kits for in house directly conjugating mAbs with fluorochromes. Directly fluorochrome conjugated anti-human and anti-bovine LDMs, with potential cross-reactivity with buffalo and mAbs directly conjugated with fluorochromes with commercial kits were used to compare use in multicolor flow cytometry. No difference was observed in the methods for multicolor flow cytometry. Indirect labeling can be used with many of the initial multicolor flow cytometric studies in buffalo minimizing the cost of reagents. Zenon second step reagents can be used where there is a need to compare labeling with mAbs of the same isotype. The kit prices are still high and add considerably to the cost multicolor flow cytometry. Where essential, kits are available for directly conjugating mAbs.

A second issue for introducing flow cytometry to the study of the immune response to pathogens in buffalo has been when and how to initiate investigations. Initial comparative studies of the buffalo immune system with cattle have shown there are similarities and differences that need to be taken into consideration when studying the immune response to common pathogens (10). We selected BVDV as a model pathogen to initiate use of flow cytometry in the study of infectious diseases in buffalo because of ongoing interest in the pathogenesis BVDV in cattle (6) and evidence that it is an important pathogen for buffalo also. We used the study to compare direct and indirect labeling techniques and gain comparative information on the effect of infection on the composition of leukocyte subsets in blood during the 1st days of infection. The simultaneous use of FCM and routine hematological analysis revealed a similar modulation of the lymphocyte frequency caused by infection with ncp BVDV-1 as in other species (6, 26). We observed a significant reduction of leukocytes, by 4 dpi, and of total lymphocytes from 3 dpi to 8 dpi; no significant variations were observed in other leukocyte subpopulations (**Table 2**). The results were similar to results reported in bovine and alpaca species (6, 26) with a significant reduction of all $CD3^+$, $CD4^+$, and $CD8^+$ T lymphocytes, $CD21^+$ B lymphocytes and NK cells at 3 and 4 dpi. No variation was observed in CD4:CD8 ratio. Our results are in accordance with Ellis et al. (6) that showed a significant decrease of WBC and lymphocytes ($CD2^+$, $CD4^+$, and $CD8^+$) in bovine peripheral blood at 3, 5, and 7 dpi with ncp BVDV. They also observed no significant variations in the CD4:CD8 ratio, which can be explained by an equal reduction in both subsets. Similarly, Gånheim et al. (29) reported that the total leukocytes, neutrophils and lymphocytes decreased significantly from day 3 from inoculation of bovine calves with BVD virus and then increased at the 9 dpi. Reduction in the frequency of $CD8^+$ and $WC1^+$ cells was observed on days 8, 11.5, and 15.5 of the study and 11.5, 15.5, and 22.5 respectively, compared to day 2.5 of the study. No differences were observed in the numbers of B lymphocytes.

CD25, a subunit of the IL-2 receptor (IL-2R), is expressed on activated cells including T lymphocytes, B lymphocytes, and monocytes. In trial 2 the percentage of cells $CD3^+/CD25^+$ showed an increase (+33%) at 8 dpi (data not shown). A significant increase in activated lymphocytes has been reported in alpacas experimentally infected at 9 dpi (26). These observations might suggest that buffaloes and alpacas, two species thought to be more resistant to BVDV, could have a stronger lymphocyte activation than in cattle (27). Comparative studies can now be conducted to confirm and extend studies to elucidate differences in the immune response to BVDV.

Brock and coworkers (28) reported that the outcome of fetal infection was dependent on the stage of gestation. In our study, although all animals did not show clear clinical signs and symptoms (e.g., diarrhea, respiratory problems, and fever), trial 1 animals incurred severe clinical complications. Buffalo-1 aborted 57 days after the infection (138 days of gestation); unfortunately, it was not possible to perform necropsy on the aborted fetus. We could not conclude that the abortion was due to the infection. Buffalo-2 died of a postpartum prolapse and her calf died 10 days after calving. Fetal death and abortion occur during BVDV infection in cattle (29) and buffalo (20). The prolapse could be the result of increased susceptibility to bacterial infections associated with BVDV infection (30). In trial 1, our results showed a more pronounced and prolonged leuko- and lymphopenic post-infection effect that might account for the observed effects (**Table 2**). However, more extensive studies are needed to follow up this possibility.

In conclusion, this preliminary study shows that the availability of an extensive set of mAbs in buffalo has increased opportunities to conduct studies on the immune response to pathogens and development of vaccines. Moreover, since in buffalo many pathological conditions such as BVD, are asymptomatic, the flow cytometric approach may be helpful in the diagnosis and/or prognosis of some conditions.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Ministry of Health (authorization n. 503/2017-PR).

AUTHOR CONTRIBUTIONS

ED: conceptualization. FG, AM, SP, FF, GD, and ED: methodology. FG, AM, SP, RS, AD, CC, MS, CG, and DV: sample collection and analysis. FG and WD: original draft preparation. AM, MS, GD, ED, FG, and WD: review and editing. All authors have read and agreed to the published version of the manuscript.

FUNDING

This work was supported by funding from the Italian Ministry of Health for IZSME 09/13 RC Bovine Virus Diarrhea Virus:

study of the dynamics of infection in buffalo (*Bubalus bubalis*) by experimental reproduction of the disease.

ACKNOWLEDGMENTS

The authors would like to thank Dott. Gian Mario De Mia (Centro di Referenza Nazionale per lo Studio delle malattie da Pestivirus e Asfivirus, CEREP-IZSUM) for advice in writing the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.574434/full#supplementary-material

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 19 October 2020
doi: 10.3389/fvets.2020.577758

On the Effect of the Temperature-Humidity Index on Buffalo Bulk Milk Composition and Coagulation Traits

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: 30 June 2020
Accepted: 02 September 2020
Published: 19 October 2020

Citation:
Costa A, De Marchi M, Battisti S, Guarducci M, Amatiste S, Bitonti G, Borghese A and Boselli C (2020) On the Effect of the Temperature-Humidity Index on Buffalo Bulk Milk Composition and Coagulation Traits. Front. Vet. Sci. 7:577758. doi: 10.3389/fvets.2020.577758

Little is known about the effects of high levels of environmental temperature and humidity on milk yield and quality in buffaloes since this species is known to be more heat tolerant than cattle. However, the distribution of sweat glands and the dark skin color can negatively affect heat tolerance. Moreover, due to increased global temperatures, concerns regarding heat stress and thermoregulation in dairy animals, including buffaloes, have been extended to the northern hemisphere. In this study, the effects of both the temperature-humidity index (THI) and the maximum daily temperature-humidity index (MTHI) were estimated on bulk milk traits, namely fat, protein, lactose, urea content, pH levels, somatic cell score, coagulation properties, and bacteria count. The dataset consisted of repeated data from 99 Mediterranean water buffalo farms, and mixed models were used for the analyses. Supporting the negative correlations observed, bulk milk fat, protein, and lactose content were significantly lower when THI and MTHI were higher. Similarly, milk pH was lower when THI and MTHI were high; however, high levels of THI or MTHI seemed to not be markedly associated with the milk's coagulation ability. According to both analysis of variance and correlations, the somatic cell score was not significantly affected by the THI and MTHI. This is the first study based on a large dataset that evaluates the impact of high temperature and humidity in Italian buffalo milk and that provides correlations with traits of interest for the dairy industry, i.e., milk acidity and coagulation ability. In general, findings show that the effects of elevated THI and heat stress on bulk milk quality in buffalo is less evident than in cattle. These preliminary results intend to open debate on the issue of heat stress in dairy buffaloes that are reared in temperate regions. Further studies should focus on individual milk and performance and should investigate the relationship between high THI and buffalo fertility, behavior, and welfare.

Keywords: THI, dairy buffalo, milk coagulation, milk quality, milk acidity, heat stress

INTRODUCTION

The effects of environmental temperature and humidity on milk-related performance, fertility, and welfare have been widely studied in dairy cattle (1–3). In particular, often these two environmental parameters are combined into a unique index, the temperature-humidity index (THI), to evaluate cow heat stress and milk losses, and to monitor the thermoregulation (3). Several formulas have been proposed to calculate THI in dairy cows (4), with the Kibler formula being denoted as the most popular one due to its simplicity and efficacy.

Little is known about the effects of high levels of temperature and humidity on milk yield and quality in buffalo since this species is known to be more heat tolerant than cattle. However, it is worth highlighting that the distribution of sweat glands and the dark color of the skin negatively affect heat tolerance in these animals (5).

Due to increased global temperatures, concerns regarding heat stress and thermoregulation in dairy animals, including buffaloes, has been extended to the northern hemisphere. Italy hosts the majority of European buffaloes (6); the water buffalo has been traditionally present and farmed in the southern regions of the country, particularly where Mozzarella di Bufala Campana cheese, which has been granted a "protected designation of origin" status, is manufactured (7).

To limit the negative impact of high THI during the summer and improve animal welfare, showers and foggers are installed in some Italian farms to reduce environmental temperatures and decrease both the respiratory rate and body temperature of buffaloes. Despite this, cheese industries still consider the summer milk to be the worst for cheesemaking.

Thus, in the present study, the effects of different levels of THI and maximum temperature-humidity index (MTHI) on milk quality traits have been estimated using data of bulk milk samples collected during the year 2018.

MATERIALS AND METHODS

Herds

Data collection was carried out in 99 commercial Mediterranean water buffalo herds located in the Latium region (Italy), in the area of Mozzarella di Bufala Campana cheese. Bulk milk sampling lasted 12 months, from January 2018 to December 2018. The geographical coordinates of each herd were obtained from the national database of the zootechnical registry established by the Ministry of Health at the National Service Center of the Experimental Zooprophylactic Institute of Abruzzo and Molise (Teramo, Italy). At least three milk samples were available for each herd.

Temperature and Humidity Index

Climatic data consisted of daily average and maximum temperatures (°C) and relative humidity, and were extrapolated from the Lazio Region Integrated Agrometeorological Service database (SIARL; http://www.arsial.it/portalearsial/Agrometeo/E7.asp). Data were recorded by 26 meteorological stations with known coordinates (latitude and longitude) and distributed between the provinces of Frosinone and Latina (Latium region), i.e., where the herds were located. Through the geographical coordinates of weather stations and herds, a spatial interpolation was performed by the "inverse distance weighted" technique using the ArcGis 10.3.1 software in order to identify the closest weather station for each herd. In ArcGis 10.3.1 software, the "extract multi values to points" technique allowed for the creation of a database where each herd in each sampling date had unique values of the daily average (AT) and maximum temperature (MT) and relative humidity (RH).

According to the literature (4, 8, 9), THI and MTHI were calculated using Kibler's equations:

$$THI = (1.8 \cdot AT) - (1.0 - RH) \cdot (AT - 14.3) + 32\,;$$
$$MTHI = (1.8 \cdot MT) - (1.0 - RH) \cdot (MT - 14.3) + 32\,.$$

In particular, the MTHI represents the worst daily environmental conditions experienced by the lactating buffaloes and was calculated based on suggestions found in the literature (9); in fact, sensitivity of test-day milk yield to MTHI was found to be greater compared with THI in cattle.

Milk Quality Determination

Milk samples were refrigerated (4°C) and analyzed within 24 to 36 h from collection at the Experimental Zooprophylactic Institute of Lazio and Tuscany (Rome, Italy), the national reference laboratory for dairy product quality in Central Italy. Content (%) of fat (FC), protein (PC), lactose (LC), milk urea nitrogen (MUN, mg/dL), pH, and electrical conductivity (EC, mS) were determined by the MilkoScan FT6000 (FOSS Electric, Hillerød, Denmark), calibrated with appropriate buffalo standards. Somatic cell count (SCC, cells/mL) and total bacterial count (TBC, CFU/mL) were recorded by Fossomatic and Bactoscan, respectively (FOSS Electric, Hillerød, Denmark). To achieve a normal distribution of the SCC data, the somatic cell score (SCS, units) was calculated as: SCS = $\log_2$ (SCC/100,000) + 3 (3). For the same reason, all values of TBC were log-transformed to LTBC, as LTBC = $\log_{10}$ (TBC). The limit fixed by the EU Regulation no. 853 of 2004 for buffalo milk TBC is 1,500,000 CFU/mL; however, this limit is reduced to 500,000 CFU/mL if the milk used to manufacture dairy products is not heat treated (10).

Milk clotting parameters were determined through reference method by the Formagraph (FOSS Electric, Hillerød, Denmark) using animal rennet according to conventional protocols (11, 12). Briefly, each milk sample (10 mL) was heated to 35°C and added with 200 μL of commercial calf rennet (75% chymosin and 25% bovine pepsin, Caglificio Clerici SPA-Sacco SRL, Cadorago, Italy) diluted (1:1) in distilled water. The milk clotting parameters recorded were:

- RCT (rennet coagulation time, min): interval between the rennet addition and beginning of coagulation;
- k_{20} (curd firming time, min): the time necessary to obtain a curd firmness of 20 mm; and
- a_{30} (curd firmness, mm): the curd firmness recorded after 30 min of rennet addition.

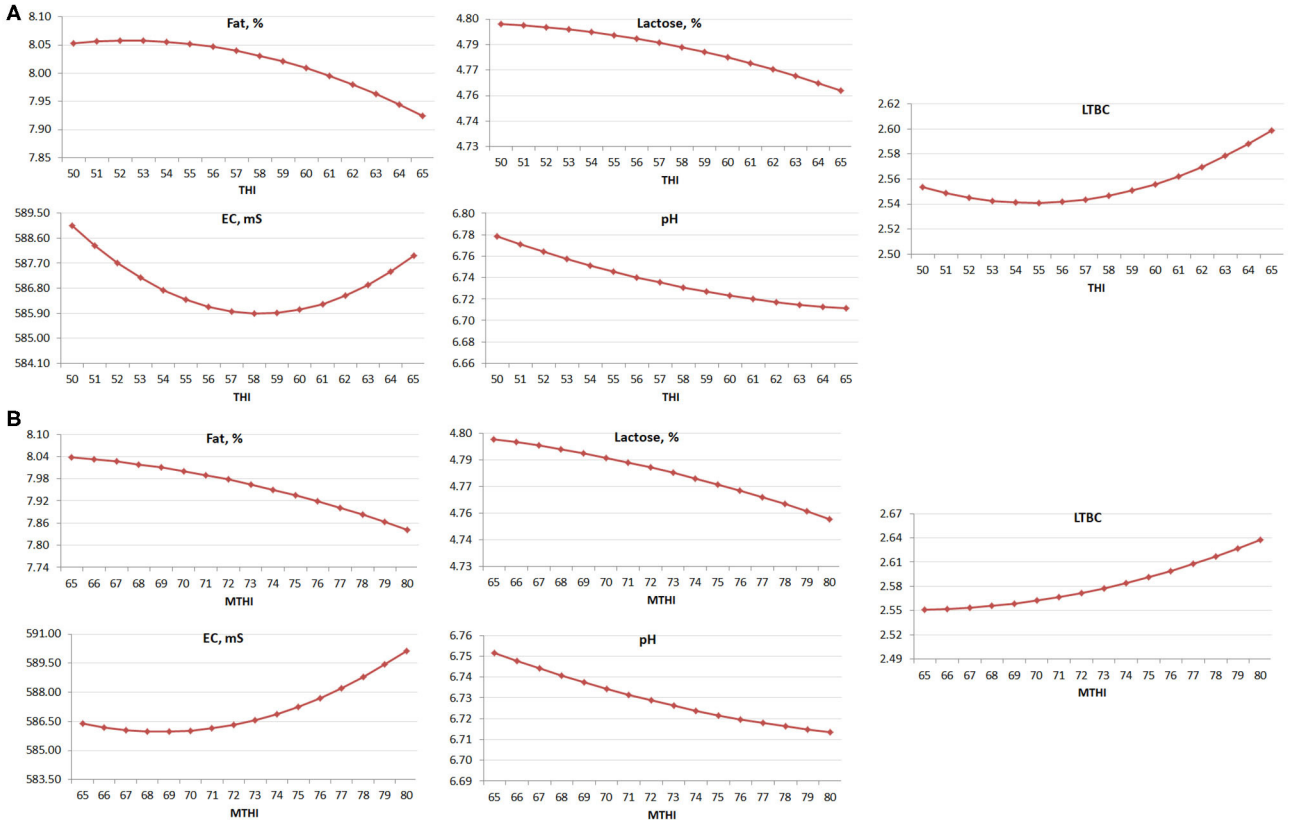

FIGURE 1 | Regression (linear + quadratic) of **(A)** THI and **(B)** MTHI on the studied milk traits ($P < 0.05$).

Statistical Analysis

Regression coefficients were obtained with a mixed model in SAS software v. 9.4 (SAS Institute Inc., Cary, NC), where THI (or MTHI) was included as linear and quadratic covariate (fixed effects) and where the farm was the random factor. The regressions were performed in order to test the significance of the effect for each milk feature (**Figure 1**). For the analysis of variance, the mean, and the standard deviation (SD) of THI (**Table 1**) were used to design three classes, as follows:

- Class 1 (low): if THI < mean – 1 SD;
- Class 2: if THI ≥ mean – 1 SD and < mean + 1 SD; and
- Class 3 (high): if THI ≥ mean + 1 SD.

At the end, classes 1, 2, and 3 of THI included 24.25, 51.58, and 24.17 % of the observations, respectively.

The following model was used to estimate the effect of the level of THI on milk quality traits in the SAS software v. 9.4 (SAS Institute Inc., Cary, NC)

$$y_{ijk} = \mu + Thi_i + Herd_j + e_{ijk},$$

where y_{ijk} is the bulk milk trait investigated (FC, PC, LC, MUN, EC, pH, SCS, LTBC, and the clotting parameters); μ is the overall intercept of the model; $Thi_i$is the fixed effect of the ith class of THI (j = 1 to 3); $Herd_j$ is the random effect of the jth herd (≥3 at least 3 bulk milk records per herd); and e_{ijk} is the random residual. A multiple comparison of least squares means (LSM) for the fixed effects was performed using the Bonferroni's test ($P < 0.05$).

TABLE 1 | Descriptive statistics of temperature-humidity index (THI), maximum of temperature-humidity index (MTHI), and milk traits in the whole dataset.

Trait	*n*	Mean	Standard deviation	Min.	Max.
THI	1,390	58.44	10.50	39.66	76.09
MTHI	1,390	68.66	12.59	45.65	91.65
Milk traits					
Fat, %	1,385	7.95	0.66	5.93	9.95
Protein, %	1,381	4.49	0.24	3.72	5.25
Lactose, %	1,374	4.78	0.13	4.35	5.22
Milk urea nitrogen, mg/dL	1,386	34.00	9.24	8.20	61.90
Electrical conductivity, mS	1,379	589.27	27.70	513.20	684.40
Rennet coagulation time, min	1,357	13.25	4.53	2.45	27.30
Curd firmness, mm	1,318	49.28	10.09	11.22	88.00
Curd firming time, min	1,323	3.96	2.63	0.20	16.30
pH	1,383	6.75	0.10	6.46	7.06
Somatic cell score	1,390	3.52	0.81	1.11	6.30
Log_{10} of total bacteria count	1,353	2.47	0.66	0.60	3.70

The same approach was used to estimate the effect of the level of MTHI on the same milk quality traits. In particular, MTHI was a fixed effect with three classes, as follows:

- Class 1 (low): if MTHI < mean – 1 SD;
- Class 2: if MTHI ≥ mean – 1 SD and < mean + 1 SD; and
- Class 3 (high): if MTHI ≥ mean + 1 SD.

The mean and SD of MTHI are given in **Table 1** and the frequency of observation in classes 1, 2, and 3 was 26.91, 53.45, and 19.64%, respectively.

RESULTS

Overall, the THI reached a maximum in August and a minimum in January (**Figure 2A**) and, on average, the THI was 58.44 while the MTHI was 68.66 (**Table 1**). The greatest MTHI value recorded by the stations was 91.65 (**Table 1**). The correlation between THI and MTHI was overall equal to 0.98 ($P < 0.001$, data not shown), but their association showed variation across the months (**Figure 2B**). In particular, the two indices were less correlated in winter.

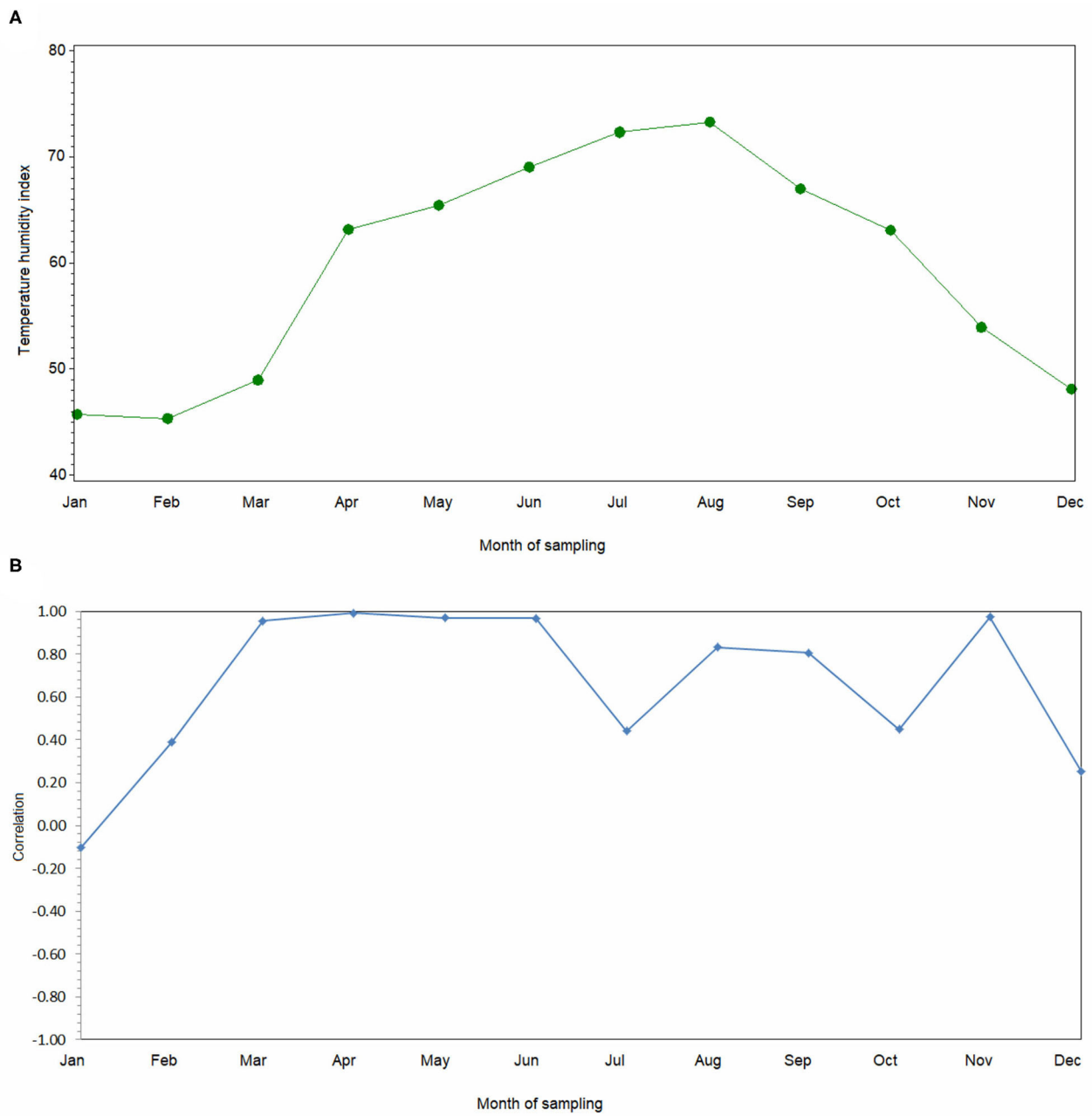

FIGURE 2 | Trend of **(A)** average temperature-humidity index and **(B)** correlation between temperature-humidity index and maximum temperature-humidity index during 2018.

TABLE 2 | Pearson correlations ($P < 0.05$) between bulk milk fat (%, FC), protein (%, PC), lactose (%, LC), urea nitrogen content (mg/dL, MUN), electrical conductivity (mS, EC), rennet coagulation time (min, RCT), curd firmness (mm, a_{30}), curd firming time (min, k_{20}), pH, somatic cell score (SCS), and log_{10} of total bacteria count (LTBC).

Trait	FC	PC	LC	MUN	EC	RCT	a_{30}	k_{20}	pH	SCS
PC	0.54									
LC	−0.21	−0.33								
MUN	ns	−0.07	−0.09							
EC	−0.33	ns	−0.36	−0.12						
RCT	0.09	0.16	ns	ns	−0.07					
a_{30}	0.11	0.08	−0.06	ns	−0.05[a]	−0.48				
k_{20}	ns	ns	ns	ns	ns	ns	−0.44			
pH	−0.46	−0.26	0.45	−0.31	0.09	0.07	−0.12	ns		
SCS	0.06	0.19	−0.19	ns	0.24	0.06	−0.06	0.05[a]	−0.06	
LTBC	−0.13	−0.16	−0.10	ns	0.17	−0.22	ns	ns	−0.12	0.06

[a] *0.05≤ P <0.10.*

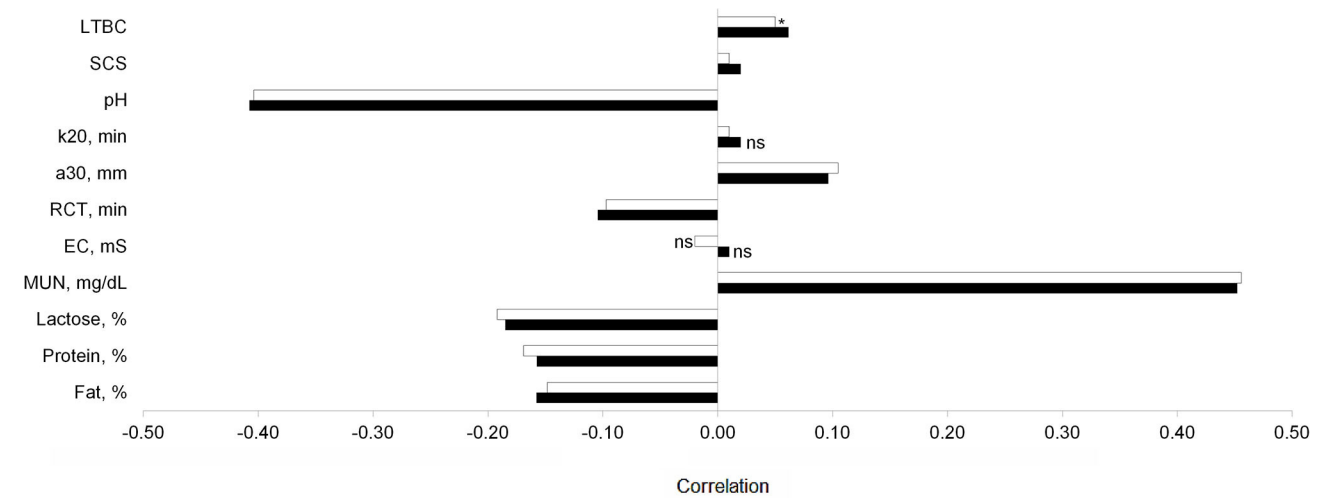

FIGURE 3 | Pearson correlations ($P < 0.05$) of temperature-humidity index (black bar) and maximum temperature-humidity index (white bar) with milk composition traits, urea nitrogen (MUN), electrical conductivity (EC), rennet coagulation time (RCT), curd firmness (a_{30}), curd firming time (k_{20}), somatic cell score (SCS), and log_{10} of total bacteria count (LTBC). Asterisk indicates $0.05 \leq P < 0.10$.

Descriptive statistics of milk composition traits are depicted in **Table 1**; according to the mean and SD of each trait, the most variable feature was k_{20} with a coefficient of variation of 66.4%, followed by RCT, MUN, and LTBC. The trait with the lowest coefficient of variation (2.7%) was LC, followed by EC and PC.

Milk quality features and coagulation traits were overall weakly to moderately correlated with each other and some associations were not significant (**Table 2**). In particular, the strongest correlations were those between FC and PC (0.54), RCT and a_{30} (−0.48), pH and FC (−0.46), pH and LC (0.45), and LC and EC (−0.33).

The correlations of THI with milk traits were similar to those of MTHI (**Figure 3**); in fact, the strongest correlations of both THI and MTHI were with MUN and pH (**Figure 3**). Both THI and MTHI were also significantly positively correlated with LTBC, although weakly.

In general, the associations with major milk solids (FC, PC, and LC) and with RCT were negative with a moderate to weak magnitude. Furthermore, the correlations of THI and MTHI with a_{30} were positive but weak, i.e., < 0.15 (**Figure 3**).

The mean, SD, min, and max for THI and MTHI for each class is presented in **Table 3**. Except for RCT, k_{20}, and SCS all milk traits studied were significantly affected by the class of THI (**Table 4**). In fact, milk in the high THI class (class 3) was characterized by the lowest solid content, i.e., FC, PC, and LC. On the contrary, the lowest LSM of MUN was observed in class 1 (**Table 4**). Milk pH was the lowest in class 3, where acidity was thus more favorable for the coagulation.

The effect of the MTHI was not significant for k_{20} and SCS (**Table 5**). The worst milk composition in terms of FC, PC, and LC was observed in class 3 (high). In the same class, LTBC and MUN were the highest (**Table 5**). Finally, milk pH was greater when MTHI was lower, i.e., in class 1.

TABLE 3 | Descriptive statistics of temperature-humidity index (THI) and maximum of temperature-humidity index (MTHI) for each class.

Class	*n*	Mean	Standard deviation	Min	Max
THI					
1 (low)	337	45.02	1.36	39.66	47.72
2	717	58.53	6.58	47.98	68.94
3 (high)	336	71.72	209	68.95	76.09
MTHI					
1 (low)	374	52.62	2.71	45.65	56.06
2	743	70.64	7.44	56.07	81.24
3 (high)	273	85.25	2.79	81.31	91.65

TABLE 4 | Significance of linear (P_L) and quadratic (P_Q) regression coefficient of temperature-humidity index (THI) and least squares means of bulk milk traits for the fixed effect of THI with significance (*P*) of the effect (three classes).

Trait	P_L	P_Q	Class of THI			
			1 (low)	2	3 (high)	*P*
Fat, %	***	***	8.00[a]	8.00[a]	7.70[b]	***
Protein, %	ns[1]	ns	4.54[a]	4.49[b]	4.36[c]	***
Lactose, %	**	**	4.80[a]	4.79[a]	4.73[b]	***
Milk urea nitrogen, mg/dL	ns	ns	27.81[c]	33.61[b]	39.18[a]	***
Electrical conductivity, mS	***	***	594.36[a]	588.56[ab]	592.67[b]	**
Rennet coagulation time, min	ns	ns	13.42[a]	13.22[a]	12.78[a]	ns
Curd firmness, mm	ns	ns	48.15[b]	49.73[a]	49.40[ab]	*
Curd firming time, min	ns	ns	3.94[a]	3.90[a]	4.10[a]	ns
pH	***	***	6.83[a]	6.74[b]	6.71[c]	***
Somatic cell score	ns	ns	3.53[a]	3.56[a]	3.51[a]	ns
Log_{10} of total bacteria count	**	**	2.61[ab]	2.58[b]	2.68[a]	*

*Different letters within row indicate statistical significance (P < 0.05). ***P < 0.001, **P < 0.01, *P < 0.05, [ns]not significant.*
[1]$P_L = 0.06$.

TABLE 5 | Significance of linear (P_L) and quadratic (P_Q) regression coefficient of maximum temperature-humidity index (MTHI) and least squares means of bulk milk traits for the fixed effect of MTHI with significance (*P*) of the effect (three classes).

Trait	P_L	P_Q	Class of MTHI			
			1 (low)	2	3 (high)	*P*
Fat, %	***	***	8.01[a]	7.97[a]	7.66[b]	***
Protein, %	ns[1]	ns	4.56[a]	4.47[b]	4.44[c]	***
Lactose, %	**	**	4.80[a]	4.79[b]	4.73[c]	***
Milk urea nitrogen, mg/dL	**	ns	27.70[c]	34.37[b]	39.38[a]	***
Electrical conductivity, mS	***	***	595.36[a]	587.98[b]	593.65[a]	***
Rennet coagulation time, min	ns	ns	13.83[a]	12.97[b]	12.78[b]	**
Curd firmness, mm	ns	ns	46.98[b]	50.10[a]	49.80[a]	***
Curd firming time, min	ns	ns	3.96[a]	3.92[a]	4.06[a]	ns
pH	***	***	6.82[a]	6.73[b]	6.72[b]	***
Somatic cell score	ns	ns	3.53[a]	3.56[a]	3.49[a]	ns
Log_{10} of total bacteria count	**	**	2.59[a]	2.60[a]	2.69[b]	*

*Different letters within row indicate statistical significance (P < 0.05). ***P < 0.001, **P < 0.01, *P < 0.05, [ns]not significant.*
[1]$P_L = 0.07$.

Considering the regression coefficients (linear and quadratic) of THI a significant effect was observed for some traits (**Figure 1A**), with the exception of PC, MUN, coagulation properties, and SCS (**Table 4**). Despite this, the linear effect of THI on PC was almost significant, with $P = 0.06$. The linear and the quadratic effects of MTHI (**Figure 1B**) were not significant for PC, coagulation properties and SCS and, in the case of MUN, only the linear effect was significant (**Table 5**). Despite this, the linear effect of MTHI on PC was close to the significance threshold, with $P = 0.07$.

DISCUSSION

Descriptive statistics observed in this study for FC, PC, LC, SCS, and coagulation traits mirrored those reported in studies based on bulk milk data collected on 36 Italian buffalo herds (11) and on 1,414,449 individual buffalo milk test-day records (7). As expected, the variability of the bulk milk traits was lower compared with individual samples (7). However, a comprehensive comparison with the literature was not possible, since buffalo bulk milk data collected on a large scale are usually scarcely available.

Pearson correlations (**Table 2**) were overall in agreement with those reported in literature for Mediterranean water buffalo and based on individual milk samples (7, 13). Moreover, some correlations, like those of THI with milk FC, PC, and LC, were in line with the trend observed for the LSM in the three classes of THI (**Table 4**). Milk samples collected in presence of high THI or high MTHI (class 3) showed more favorable coagulation properties but a lower concentration of solids compared with class 1 (**Tables 4**, **5**). To our knowledge only few studies have investigated the effects of THI on milk quality traits in buffalo worldwide and, overall, FC usually is affected by THI. In particular, a reduction of about 13% in milk FC has been observed between the cold and hot season (14) in individual milk. In this study, a considerable decrease of FC was observed moving from class 1 and 2 to class 3 (**Tables 4**, **5**). In the case of Egyptian and Murrah buffaloes (14, 15), authors have reported a significant reduction of milk FC and of all solids content with increasing environmental temperature. The relationship between THI and milk quality traits has been recently investigated in 51 bulk milk samples (16); the results confirmed those of the present study, evidencing the greatest FC (8.57%) in January and the lowest (6.50%) in the summer season. On the opposite, PC was not significantly affected by the season of sampling (16, 17).

Concerning SCS and LTBC, the effect of THI and MTHI was not clear; this was also confirmed by the weak correlations (**Figure 3**). In the same area, the variation of SCC (average of 137,692 cells/mL) and bacteria count (average of 251,013 UFC/mL) across months was investigated (18); authors found not-significant variation for SCC, while the bacteria count increased during the warm months.

Both THI and MTHI significantly affected MUN, with more favorable (lower) LSM in class 1 (**Tables 4**, **5**). These results were consistent with a study investigating the variation of serum and milk urea in the presence of heat stress in buffaloes (19). In particular, the serum urea concentration recorded in July was significantly greater than the one recorded in May, likely because of an increase in gluconeogenesis, the catabolism of amino acids and rumen ammonia levels during hot and humid periods.

In regard to coagulation traits, some correlations and LSM apparently seem to suggest a favorable association between a_{30} and THI and between RCT and MTHI; however, it is important to highlight that from a cheese making point of view, shorter RCT and greater a_{30} are desirable, but in some cases this situation corresponds to a compromise between milk acidity and microbiological profile. Milk k_{20} was not affected by either THI or MTHI.

CONCLUSIONS

In this study the effects of THI and MTHI were estimated on bulk milk quality traits in Mediterranean water buffalo. This is the first study using a large dataset to evaluate the impact of high environmental temperature and humidity in this species in Italy and to provide correlations of traits of interest for the dairy industry, including clotting parameters. In general, the findings show that the effects of heat stress on buffalo milk quality traits are less evident than in cattle and, therefore, comparisons and parallelisms with studies dealing with the effects of THI in bovines may be not appropriate. Milk solids content was impaired by high THI and MTHI; in fact, both FC and PP, which are important for cheese making, were significantly lower when the temperature and humidity were higher, and this was supported by the negative correlations observed.

Milk pH was lower and more favorable when THI and MTHI were high, however, on the other hand, the impact of high levels of THI or MTHI on the three coagulation properties was not clear. Specific strategies based on precision livestock farming techniques should be studied to reduce the impact of heat stress in the Italian buffalo.

This study should be therefore considered preliminary, with the purpose of opening the debate in the scientific community on the issue of heat stress in dairy buffaloes that are reared in the northern hemisphere. Further studies should focus on individual milk and explore the relationship between high THI and animal reproductive performance, behavior, and welfare.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study because milk data routinely available were used and thus no interaction with animals was present. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

The work was conceived and designed by CB, AB, and MD. SB, MG, SA, and GB were in charge to set up the experimental design and to follow milk sampling. AC and CB performed the analyses and interpreted the results. The manuscript was mainly written by AC, CB, MD, and AB. All authors have approved the final version.

FUNDING

This paper is based on the research project IZSLT 07/19 (G81F19000190001) funded by the Italian Ministry of Health (Rome, Italy).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

REVIEW
published: 28 October 2020
doi: 10.3389/fvets.2020.557967

Cryptosporidiosis and Giardiasis in Buffaloes (*Bubalus bubalis*)

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Cryptosporidium spp. and *Giardia duodenalis* infect the gastrointestinal tracts of animals and humans. Both parasite groups are distributed worldwide and cause significant economic losses in animal productivity. Infected hosts presenting with and without clinical manifestations can eliminate infective forms of these protozoa, which are particularly important to One Health. Compared to the published research on cattle, relatively few studies have examined the epidemiology of cryptosporidiosis and giardiasis in buffaloes. This short review describes the global occurrence of *Cryptosporidium* spp. and *G. duodenalis* in buffaloes, including the molecular techniques employed for the identification of species/assemblages and genotypes of these protozoa. Genetic analyses of isolates of *G. duodenalis* and *Cryptosporidium* spp. from various sources (environmental, animal, and human) have been performed to investigate their epidemiology. In buffaloes, the species *Cryptosporidium parvum*, *Cryptosporidium ryanae*, *Cryptosporidium bovis*, and *Cryptosporidium suis*-like have been characterized, as well as assemblages A and E of *G. duodenalis*. We demonstrate that buffaloes can be infected by species of *Cryptosporidium* spp. and *G. duodenalis* assemblages with zoonotic potential. Epidemiological studies that utilize molecular biology techniques represent an important resource for efforts to control and prevent the spread of these protozoans.

Keywords: ***Cryptosporidium*, *Giardia*, water buffalo, zoonosis, genotypes, assemblages**

OPEN ACCESS

Edited by:
Ferran Jori,
UMR ASTRE—CIRAD, France

Reviewed by:
David Carmena,
Carlos III Health Institute (ISCIII), Spain
Hans-Peter Fuehrer,
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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *01 May 2020*
Accepted: *16 September 2020*
Published: *28 October 2020*

Citation:
de Aquino MCC, Inácio SV, Rodrigues FdS, de Barros LD, Garcia JL, Headley SA, Gomes JF and Bresciani KDS (2020) Cryptosporidiosis and Giardiasis in Buffaloes (Bubalus bubalis). Front. Vet. Sci. 7:557967. doi: 10.3389/fvets.2020.557967

INTRODUCTION

Cryptosporidium spp. and *Giardia duodenalis* (synonym: *Giardia lamblia*, *Giardia intestinalis*) are unicellular protozoan parasites that infect the intestinal tracts of humans and animals (1–3). Although these parasites possess biological differences, they are frequently discussed together because they share transmission pathways and cause diseases in the gastrointestinal tract (4).

Oocysts and cysts are transmitted *via* the fecal–oral route following direct or indirect contact with the transmissible stages. Possible propagation mechanisms of *Cryptosporidium* spp. and *G. duodenalis* include from animal to animal, from person to person, through zoonotic transmission, *via* contaminated food (5), by water delivery through drinking water, and in the course of

recreational activities (6). Insects can also act as mechanical carriers of these protozoa (7). These parasitic forms of *G. duodenalis* and *Cryptosporidium* spp. remain infectious for months in environments with favorable temperature and humidity conditions, which enables the perpetuation of the biological cycle and parasitic spread (4).

Ruminants are frequently considered a major source of excretion of *Cryptosporidium* spp. and *G. duodenalis* for humans (8, 9). Although most studies on this topic have investigated cattle, water buffaloes can also become infected and excrete *Cryptosporidium* spp. oocysts (**Table 1**) and *G. duodenalis* cysts (**Table 2**). In general, young buffaloes are more affected by these agents than are older animals (11, 16, 18, 25, 27, 34).

Cryptosporidium spp. and *G. duodenalis* cause mild and/or moderate disease, with diarrhea being the main clinical sign. There are few reports describing the economic burden of *Cryptosporidium* spp. and *G. duodenalis* infection in ruminants. The costs of treatment, reduced feed conversion, production inefficiency, and the involvement of many animals in the herd cause considerable economic losses on farms worldwide (25, 35). Dairy calves determined to be negative for *Cryptosporidium* or *G. duodenalis* by immunofluorescence microscopy showed higher average daily gain than did calves that were positive for these parasites (36).

The application of molecular approaches for the identification of these two parasites has led to significant advances in knowledge regarding the epidemiology of these protozoans, with different species being characterized (37).

Molecular analysis of human and animal isolates has demonstrated that *G. duodenalis* is a complex species, with eight assemblages being recognized. Assemblages A and B are observed in humans and other mammals, assemblages C and D are specific to dogs and other canids, assemblage E is found in hoofed animals, including livestock, assemblage F is detected in cats, assemblage G is found in rodents, and assemblage H is observed in pinnipeds (38).

To date, at least 38 species of *Cryptosporidium* spp. have been recognized by molecular characterization (39–41), with 73 genotypes (42–44) and 17 species having been identified in humans (45).

Cryptosporidium spp. and *G. duodenalis* may represent a problem to the buffalo industry due to their economic cost and the risk of human exposure associated with oocysts and cysts eliminated in the environment by infected hosts. Thus, in this

TABLE 1 | Occurrence of *Cryptosporidium* spp. in buffaloes worldwide using different diagnostic techniques.

Country	Study period	Number of animals	Animal age (months old)	Positive numbers of buffaloes according to the diagnostic technique				
				Diagnostic method	Microscopy	Immunologic	PCR	References
Italy	NA	57	-	ELISA/IFA/PCR	-	8	6*	(10)
Italy	2006	347	<1	ELISA	-	51	-	(11)
India	2009–2010	162	<5	ZN/ PCR	62	-	62*	(12)
Nepal	2010	81	2–7	ZN/ PCR	30	-	16*	(13)
Egypt	2010–2011	538	≤4–>4	ZN/ PCR	17	-	17*	(14)
India	2009–2010	113	<6	PCR	-	-	41	(15)
Egypt	2011	211	≤1–≥1	TRQ/PCR	-	43	75[a]	(16)
India	2009–2012	264	<3	ZN/ PCR	64	-	16[a]	(17)
Australia	NA	476	≤24	PCR	-	-	62	(18)
Sri Lanka	2012–2013	297	<6–≥6	PCR	-	-	29	(19)
Thailand	2010–2011	600	≤3–≥3	DMSO-MAFS/PCR	34	-	34	(20)
Egypt	2010–2011	466	≤3–≥3	ZN/ PCR	6	-	6*	(21)
Chine	2012	181	-	PCR	-	-	43	(22)
Brazil	2010	222	5≤6	PCR	-	-	107	(23)
India	2012–2013	246	≤3–≥3	ZN/ PCR/ S	91	-	6[a]	(24)
Egypt	2014	130	<2–>12	ZN/ PCR	16	-	4[a]	(25)
Australia	2015	100	24≤60	qPCR	-	-	21	(26)
India	2014–2016	83	≤3–≥3	/PCR	-	9	1*	(27)
Brazil	NA	122	<12	Nested PCR/RFLP	-	-	16	(28)
Australia	2017	313	-	PCR	-	-	42	(29)

**PCR was performed only on positive samples by a previous screening method.*
[a]PCR was performed on only some of the positive samples by the screening method.
PCR, Polimerase Chain Reaction; ZN, Ziehl-Neelsen; ELISA, Enzyme-Linked Immunosorbent Assay; IFA, Immunofluorescence Assays; TRQ, Test RIDA®QUICK; DMSO- MAFS, DMSO-DMSO- modified acid-fast stain; IM, Imunofluorescent Microscopy; S, Sheather.
NA, not available.

TABLE 2 | Occurrence of *G. duodenalis* in buffaloes worldwide using different diagnostic techniques.

Country	Study period	No of. animals	Animal age (months)	Positive numbers of buffaloes according to the diagnostic technique				
				Diagnostic method	Microscopy	Immunologic	PCR	References
Italy	NA	57	-	ELISA/IFA/PCR	-	15	8*	(10)
Italy	2006	347	<1	ELISA	-	63	-	(11)
Australia	NA	476	≤24	PCR	-	-	62	(18)
Sri Lanka	2012–2013	297	<6–≥6	PCR	-	-	2	(19)
Egypt	2011	211	≤1–≥1	Test RIDA®QUICK/PCR	10	-	10	(30)
India	2012	22	-	IMS/DFA	-	9	-	(31)
Egypy	2013–2014	100	1<4	DSM/ FEST / S/PCR	25	-	25	(32)
India	2014–2016	83	≤3–≥3	IM/PCR	-	9	1	(27)
Brazil	2016	183	≤6	PCR	-	-	12	(33)
Australia	2017	313	-	PCR	-	-	14	(29)

**PCR was performed only on positive samples by a previous screening method.*
PCR, Polymerase Chain Reaction; IMS, Immunomagnetic separation; DFA, Direct immunofluorescence; ELISA, Enzyme-Linked Immunosorbent Assay; IFA, Immunofluorescence Assays; Direct smear method; Formalin-ether sedimentation technique; IM, Immunofluorescent microscopy; S, Sheather; RIDA®QUICK. NA, not available.

review, we demonstrate the global occurrence of these protozoa, emphasizing the importance of the molecular characterization of their species/assemblages and genotypes reported in buffaloes.

DIAGNOSTIC METHODS

Cryptosporidiosis and giardiasis can be diagnosed by a wide variety of parasitological, serological, and molecular techniques (4, 46–48).

Regarding the parasitological diagnosis technique, the identification of the morphological structures of the *Cryptosporidium* spp. oocysts and *G. duodenalis* trophozoites or cysts is important, with *G. duodenalis* being diagnosed by direct microscopic observation of the trophozoites or cysts in feces. Trophozoites can be observed by direct microscopic examination of freshly collected samples, which are immediately prepared with saline solution at 37°C (49). Concentration techniques, such as zinc sulfate (50), sucrose (51), formalin (52), and the "three fecal test" (TF test) (53), are recommended before the observation of cysts since these methods promote an increase in diagnostic sensitivity. Centrifugal flotation with zinc sulfate is one of the most commonly employed methods for the detection of *G. duodenalis* from fecal samples; however, sucrose flotation works adequately well, and it is generally employed in ruminant samples since oocysts of *Eimeria* spp. and *Cryptosporidium* spp. may also be encountered (4).

Various staining procedures can be used to differentiate between *Cryptosporidium* spp. oocysts and *G. duodenalis* cysts from coexisting protists and for excluding similarities from environmental or fecal debris. Smear preparations stained by the trichome and iodine or iron hematoxylin methods can be utilized to assist in the detection of various stages of *G. duodenalis* (49). The most frequently used routine techniques for examining stained slides to identify *Cryptosporidium* spp. are the modified Ziehl–Neelsen (54, 55), modified Kinyoun (56, 57), and methylene blue safranin (58, 59) stains. Additionally, the negative coloring observed with the malachite green technique (53, 60) can be used. The centrifugal flotation technique with Sheather's solution (61), which uses brightfield or phase contrast optical microscopy, can be employed to visualize oocysts (8). However, differentiating between *Cryptosporidium* species/genotypes using microscopy is not possible because oocysts are similar in shape and overlap in size (37).

Immunological methods have higher sensitivity and specificity than light microscopy in characterizing diverse types of samples (49, 62, 63). Monoclonal antibodies targeting antigens in fecal samples are sensitive diagnostic methods (64, 65). Antigens of *Cryptosporidium* spp. and *G. duodenalis* in fecal contents can be detected by direct immunofluorescence assay (63, 66), ELISA (67–69), and rapid solid-phase qualitative immunochromatography assays (67). Some immunoenzymatic tests can be employed to detect infection in animals that are not eliminating cysts in the feces (70), which means that these tests can be utilized to screen large numbers of animals rapidly (67). However, these methods have the disadvantage of not being able to identify species or genotypes (45).

The application of molecular techniques has resulted in expanded knowledge regarding the taxonomy and epidemiology of *Cryptosporidium* spp. and *G. duodenalis*. Molecular diagnostics are widely used to differentiate *Cryptosporidium* spp. and *G. duodenalis* species or genotypes (35). Therefore, such methods as polymerase chain reaction (PCR), real-time PCR, or multiplex PCR together with DNA sequencing can identify species/assemblages with high sensitivity and specificity, and these techniques can be employed to identify sources of transmission as well as the zoonotic potential of these two parasites (71–73).

Genotyping targeting the small subunit of the ribosomal RNA gene (*ssu* rRNA) aligned with PCR and sequencing

the restriction fragment polymorphism (RFLP) are sensitive molecular tools used in the detection of *Cryptosporidium* spp. DNA in fecal and environmental samples (74, 75). Other genetic regions have also been studied, such as the 70-kDa heat shock protein (*hsp*70), thrombospondin-related adhesive proteins, *Cryptosporidium* spp. oocyst wall protein (*cowp*), and actin genes (76).

The 60-kDa glycoprotein (*gp60*) is located on the surface of the apical region of invasive stages of the parasite. This gene is a highly polymorphic marker that is widely used in *Cryptosporidium* spp. subtyping because of its high polymorphism and relevance to parasite biology, and the use of *gp60* subtyping has identified human-specific, animal-specific, and zoonotic subtypes for *Cryptosporidium parvum* species-specific subtypes (37). More than 20 *gp60* genotype families of *C. parvum* have been described, including several subtypes within each family, with families IIa and IId being identified from ruminants and humans and recognized as zoonotic. The IIa family and subtype IIaA15G2R1 are frequently identified in calves (3, 77).

Many genetic markers have been employed for the molecular characterization of *G. duodenalis* using degenerated primers, such as the *ssu* rRNA, glutamate dehydrogenase (*gdh*), elongation factor 1-alfa (*el1-α*), triose-phosphate isomerase (*tpi*), or genes uniquely associated with the parasite, such as beta-giardin (*bg*) (78). The *ssu* rRNA gene is strongly conserved and can be utilized to identify *G. duodenalis* assemblages, but it is of little use for studies where genetic variation within assemblages needs to be determined (78). Consequently, the *tpi*, *bg*, and *gdh* sequences are employed because polymorphism enables the characterization of the genetic heterogeneity of this parasite (79) as well as its zoonotic potential (78, 80). However, molecular methods are mostly restricted to research and specialized laboratories (37).

EPIDEMIOLOGY

Infections by *Cryptosporidium* spp. and *G. duodenalis* in water buffalo (*Bubalus bubalis*) have been less thoroughly well-investigated compared with other bovid species. **Tables 1**, **2** summarize the occurrence of these protozoa in different buffalo populations.

According to the findings described in **Table 1**, the infection rates of cryptosporidiosis reported in buffaloes ranged from 1.3% (Egypt) to 48.2% (Brazil). However, the epidemiology of giardiasis has been more heavily studied in cattle, with few reports describing *G. duodenalis* infections in buffaloes (**Table 2**), and a prevalence ranging from 1.3% (Sri Lanka) to 10.5% (India) was reported.

Differences in the prevalence of infection for *Cryptosporidium* spp. and *G. duodenalis* in buffaloes are common among epidemiological studies. The discrepancies in the reported infection rates may be attributed to the differences in environmental conditions, management practices, time between collection and processing of fecal samples, the diagnostic techniques used, age, and the number of animals analyzed in each study.

High population density, with more than 100 buffaloes being raised on a farm, intensive management systems where buffalo calves are reared at high density (11), poor or inadequate hygiene, concrete floor (81), canalized or underground water sources (16, 81), and the winter period (24, 81), as well as rainy periods (17), were relevant risk factors associated with the occurrence of infection by *Cryptosporidium* spp.

In preweaned calves, *C. parvum* causes high morbidity associated with profuse diarrhea, lethargy, anorexia, and dehydration (82). The clinical signs are evident 3–5 days after infection, and the duration of the clinical signs can vary between 4 and 18 days (83). Diarrhea is accompanied by the excretion of large amounts of oocysts, which can be subsequently dispersed within the environment (82).

Some studies have observed an association between infection by *Cryptosporidium* spp. and diarrhea in buffalo calves (17, 24, 34, 81), with a higher occurrence of infection being observed in the 1st months of age (24, 25, 34).

In calves of cattle and buffaloes from the Mumbai region of India, diarrheic feces showed a higher prevalence of *Cryptosporidium* spp. than did apparently normal feces. The highest prevalence was observed in the youngest group, declining gradually with advancing age, with the lowest prevalence being observed in adults, indicating an inverse correlation between the prevalence rate and age of the host (24). The presence of *Cryptosporidium* spp. oocysts in the feces of water buffaloes without clinical signs was verified at 6 weeks of age, which suggests that asymptomatic individuals are potential sources of infection (84).

Mixed infections by *Cryptosporidium* spp. and *G. duodenalis* in buffalo were detected by real-time PCR in 36% of animals (cattle and buffaloes) from Egypt (30). These parasites may be detected together in calves with diarrhea, and coinfection with other pathogens has also been reported (84).

In Egypt, the occurrence of *Cryptosporidium* spp. was 9.5% (17/179) in calves of buffaloes and absent in 359 adult water buffaloes (14). In some studies, *C. parvum* was identified only in buffaloes <6 months of age (18, 34). However, in Northeast Australia, all samples positive for *C. parvum* were isolated from adult buffaloes (2–5 years), indicating that this species can also commonly infect adult buffaloes (26). In Thailand, *C. parvum* infection was identified in all age groups, with no significant difference in the infection rates being observed among the age groups assessed (20).

The epidemiology of giardiasis has been more studied in cattle, and there are few reports of *G. duodenalis* infections in buffaloes (**Table 2**). In Pakistan, 2.7×10^2 cysts per gram of feces of *G. duodenalis* were excreted in fecal samples with normal consistency. Additionally, in cases of diarrhea, the intensity of elimination was higher (4.3×10^3 cysts per gram of feces). In that same study, buffalo calves (≤1 year) had significantly higher cyst prevalence (85).

The prevalence of *G. duodenalis* in Australia was 13% (62/476) with the identification of assemblages A (11.8%) and E (1.2%), respectively.

In an overpopulated province of Egypt, *G. duodenalis* cyst/trophozoites were observed in 20% of fecal samples from

people and in 25% of buffalo calves by nested PCR. Contact with calf manure and inappropriate personal hygiene practices, such as hand washing and changing shoes after handling the animals, were considered to be risk factors significantly associated with giardiasis (32).

MOLECULAR ASPECTS

Molecular diagnostic tools play an important role in understanding the transmission of *Cryptosporidium* spp. and *G. duodenalis*, mainly due to the existence of many morphologically identical species and genotypes within both groups of protozoa. Thus, these tools are required for the differentiation of these species and genotypes (86).

Although the molecular epidemiology of *Cryptosporidium* spp. and *G. duodenalis* is well-studied in cattle, studies are scarce in buffaloes. Cattle are commonly infected by four species of *Cryptosporidium*, with *C. parvum* being predominant in preweaned calves, *Cryptosporidium bovis* and *Cryptosporidium ryanae* predominant in the postweaning phase, and *Cryptosporidium andersoni* predominant in adults (45, 75, 87). However, differences in the occurrence of different species according to age groups in buffaloes were not observed.

Previous studies have determined the species of *Cryptosporidium* infecting buffaloes in different countries; *C. parvum*, *C. ryanae*, *C. bovis*, and a genotype similar to that of *Cryptosporidium suis* were identified (**Table 3**). Phylogenetic analysis using a range of sequences of the *ssu* rRNA gene of *Cryptosporidium* spp. retrieved from the GenBank database from various geographical regions supports the classification of the species and genotypes of *Cryptosporidium* spp. in buffaloes (**Figure 1**).

Cryptosporidium parvum is the most frequently reported zoonotic species that infects humans and ruminants worldwide (8). Numerous studies have been conducted to subtype *C. parvum* in farm animals, especially calves, to characterize the transmission dynamics and zoonotic potential of *C. parvum*. **Table 3** shows that most of the studies using *gp60* and sequencing analysis have observed that buffaloes are commonly infected with the IIa family. Additionally, family IId has been found in buffaloes in Egypt (14, 21, 25, 30) and India (27).

Cryptosporidium ryanae infects ruminants and has been widely reported in calves from different age groups worldwide (13, 14, 18, 19, 22–24, 28, 29). Data from recent studies suggest that there is a host-adapted *C. ryanae* in water buffaloes, as reflected by the high occurrence of this species in these animals (13, 23, 28). Some findings show that the genotypes of *C. ryanae* found in water buffaloes are different from those identified in cattle (13, 18, 22, 23). Therefore, further research is warranted to determine the taxonomic status and host specificity of these genotypes found in water buffaloes (19).

Cryptosporidium bovis is predominantly a parasite of livestock and has been reported in buffaloes on several occasions (16, 18, 22, 26, 27). Due to the lower occurrence of this species compared

TABLE 3 | Species and subtypes of *Cryptosporidium* in buffaloes worldwide.

Country	Number of animals	Genes	Species of *Cryptosporidium*	*gp60* genotype	References
Italy	57	*ssu rRNA*	*C. parvum*	-	(10)
India	162	*ssu rRNA*	*Cyptosporidium* spp.	-	(12)
Nepal	81	*ssu rRNA*	*C. ryanae*	-	(13)
India	113	*ssu rRNA*	*Cyptosporidium* spp.	-	(15)
India	264	*ssu rRNA*	*C. parvum*	-	(17)
Australia	476	*ssu rRNA*	*C. bovis. C. parvum. C. suis like. C. ryanae*	-	(18)
Egypt	211	*ssu rRNA, gp60*	*C. bovis.C. parvum. C. ryanae*	IIaA15G1R1(1). IIdA20G1 (1)	(16)
Egypt	538	*ssu rRNA, gp60*	*C. parvum. C. ryanae* (10)	IIaA15G1R1 (5). IIdA20G1 (2)	(14)
Egypt	466	*ssu rRNA, gp60*	*C. parvum. C. ryanae*		(21)
Sri Lanka	297	*ssu rRNA, gp60*	*C. ryanae. C. suis like*	-	(19)
Thailand	600	*ssu rRNA*	*C. parvum.C. ryanae*	-	(20)
Chine	181	*ssu rRNA*	*C. bovis. C. ryanae*	-	(22)
Brazil	222	*ssu rRNA, gp60, cowp*	*C. parvum. C. ryanae. C. suis like*	IIaA15G2R1(2)	(23)
Egypt	130	*ssu rRNA, gp60, cowp*	*C. parvum*	IIdA20G1 (4)	(25)
India	246	*ssu rRNA*	*C. parvum. C. ryanae*	-	(24)
Australia	100	*ssu rRNA, gp60*	*C. bovis. C. parvum*	IIaA18G3R1(5) IIdA19G1 (4). IIdA15G1 (1)	(26)
India	83	*ssu rRNA, gp60*	*C. bovis. C. parvum*	IIdA15GR1 (1)	(27)
Brazil	122	*ssu rRNA, gp60*	*C. parvum. C. ryanae*	IIdA20G1R1 (4)	(28)
Australia	313	*ssu rRNA*	*C. ryanae*	-	(29)

ssu rRNA, Small Subunit of the ribosomal RNA gene; gp60, 60 kDa glycoprotein gene; cowp, Cryptosporidium spp. oocyst wall protein.

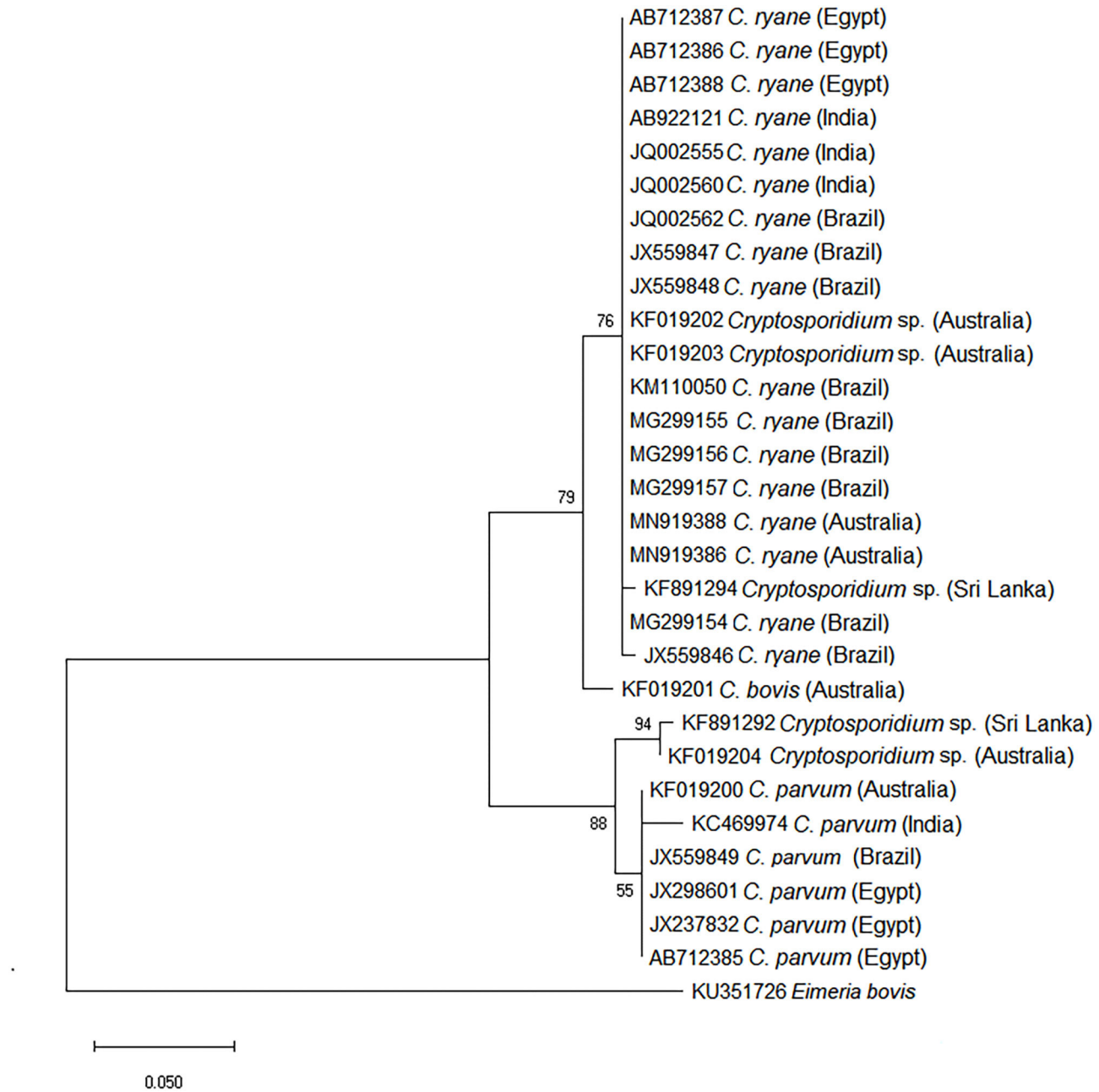

FIGURE 1 | Phylogenetic tree of species of *Cryptosporidium* using the *ssu* rRNA gene obtained from buffalo samples. The evolutionary history was inferred by using the maximum likelihood method and the Tamura–Nei model using MEGA software.

to other species of *Cryptosporidium* spp. reported in buffaloes, the possibility of a higher affinity of *C. bovis* for *Bos taurus* or *Bos indicus* was considered than for *B. bubalis* (18).

The *ssu* rRNA sequences of *C. suis*-like obtained from buffaloes in Brazil (23), Australia (18), and Sri Lanka (19) were similar to those previously identified in cattle (88–90). Based on the analysis of other genetic markers, such as *hsp* 70 and *actin*, it was observed that the sequences generated were clearly different from those of *C. suis* (23).

Regarding *G. duodenalis* infection in buffaloes, the first molecular characterization of *G. duodenalis* was performed in Italy with the detection of assemblage E and assemblage A, subassemblage AI. Based on this finding, buffaloes were suggested to contribute to environmental contamination due to the elimination of potentially infectious parasitic cysts in humans (10).

There are a limited number of studies on giardiasis in buffaloes, and in most of these, the dominant assemblage

identified was assemblage E (**Table 4**); however, zoonotic assemblage A was reported to be predominant in buffaloes from Australia (18). The predominance of this assemblage was unexpected (18).

Few studies have typed assemblage A isolates in buffaloes, but subassemblage AI was the most common (10). Studies on the prevalence of *G. duodenalis* in cattle observed the existence of different subtypes of assemblage E identified by the multilocus genotyping scheme (MLG) based on the *bg*, *gdh*, and *tpi* genes (91, 92). The differences in the distribution of *G. duodenalis* assemblage E MLGs from cattle likely indicate geographical segregation (91).

PUBLIC HEALTH IMPACT

Due to the close relationship of these protozoans with poor basic sanitation and low population purchasing power, *Cryptosporidium* spp. and *G. duodenalis* were included in the World Health Organization's Neglected Diseases Initiative (93).

Water transmission of *Cryptosporidium* spp. and *G. duodenalis* is particularly important (94), with outbreaks being reported in several countries (95, 96). Most people in undeveloped countries do not have access to good quality water. Consequently, the contamination of drinking and bathing water with these two pathogens and the use of sewage for agricultural purposes pose a serious threat to millions of individuals worldwide (97).

The transmission of *Cryptosporidium* spp. and *G. duodenalis* has also been associated with the consumption of contaminated food due to the use of fertilizers based on animal feces (manure), the use of untreated water for the irrigation of farmland, and runoff from feedlots. In addition, food can be contaminated during harvesting, packaging, transportation, and preparation under unhygienic conditions (98).

Human infections by *Cryptosporidium* spp. are commonly caused by *Cryptosporidum hominis* and *C. parvum* (1, 99), although other species have previously demonstrated zoonotic potential, such as *Cryptosporidum ubiquitum* (100), *Ctenocephalides canis* and *Ctenocephalides felis* (101–103), *Crytosporidium muris* (104, 105), *C. suis* (106), *Crytosporidium cuniculus* (107, 108), and *Crytosporidium meleagridis* (109–111).

Assemblages A and B of *G. duodenalis* can be observed in both humans and animals. Less frequently, other *G. duodenalis* genotypes have been only occasionally detected in humans (112, 113), with the possible exception of assemblage E, which has recently been detected in substantial numbers of human cases in rural areas of Egypt (25, 30, 114), Brazil (115), and Australia (116).

Cattle are recognized as a major contributor to zoonotic sources because the species and genotypes of *Cryptosporidium* spp. and *G. duodenalis* that infect humans have also been isolated from cattle (35).

Especially in developing countries, these parasites cause diarrhea in malnourished children under 5 years of age (117). Cryptosporidiosis in immunocompetent individuals is considered self-limiting, but with the appearance of the acquired immunodeficiency syndrome (HIV), opportunistic infections have been associated with more serious and even fatal clinical manifestations in immunosuppressed individuals (35, 118, 119).

The common occurrence of *C. parvum* subtypes IIaA15G1R1 and IIdA20G1 in buffaloes and humans in Egypt, respectively (16, 25), highlights the importance of zoonotic transmission with a special emphasis to the potential role of these animals as significant reservoirs of infection to humans.

In humans, most of the infections related to *G. duodenalis* are asymptomatic; however, acute or chronic diarrhea, dehydration, abdominal pain, nausea, vomiting, and weight loss can occur (120, 121). Most cases occur in individuals who are under 5 years of age, malnourished, and immunocompromised (80). Functional intestinal disorders, such as irritable bowel syndrome, can be associated with a previous infection (122). Infected children may show developmental delays, decreased cognitive function and nutritional status (123).

Among the assemblages of *G. duodenalis*, assemblages A and B have the broadest host specificity, having been known to infect humans and various other mammals (124). Both assemblages can

TABLE 4 | Assemblages and sub-assemblages of *G. duodenalis* in buffaloes worldwide.

Country	Study period	Number of animals	Animal age (months)	Genes	Assemblages	Sub-assemblages	References
Italy	NA	57	-	*bg*	A. E	A1	(10)
Australia	NA	476	A/c	*tpi*	A. E	-	(18)
Sri Lanka	2012–2013	297	<6–≥6	*tpi*	E	-	(19)
Egypt	2011	211	A/c	*bg. gdh. tpi*	E	-	(30)
India	2012	22	-	*gdh*	-	-	(31)
Egypt	2013–2014	100	1–4	*ssu* rRNA	-	-	(32)
India	2014–2016	83	A/c	*ssu* rRNA. *tpi*	A	-	(27)
Brazil	2016	183	≥6	*ssu* rRNA. *bg. gdh. tpi*	E	-	(33)
Australia	2017	313	-	*bg. gdh*	E	-	(29)

ssurRNA, Small Subunit of the ribosomal RNA gene; bg, β-giardin gene; gdh, Glutamate dehydrogenase gene; tpi, triosephosphate isomerase gene; A/c, Adults/calves; NA, not available.

be transmitted zoonotically, indicating a significant public health impact, and there are reports of farmers being infected with these assemblages (37). However, assemblage B has not been reported in buffaloes (124).

Assemblage E is considered species-specific; however, there are reports of humans being infected with this assemblage, and it was suggested that this assemblage might present an emerging anthropozoonotic cycle (30, 114–116, 125).

In Ismailia, one of the most densely populated provinces of Egypt (regarding livestock and people), fecal samples from children, cattle, and buffaloes were examined for the detection of *G. duodenalis* coproantigens and analyzed by MLG (*bg*, *gdh*, and *tpi*). Assemblage B was dominant in humans, while assemblage E was more frequent in ruminants and was detected in two children, indicating a potential route for anthropogenic infection. It was also observed that drinking tap water, but not contact with animals, was associated with an increased risk for children to be infected (30).

In Egypt, the contact of people with buffaloes and their feces was identified as a risk factor for the prevalence of infection by *G. duodenalis*, drawing attention to zoonotic transmission (32).

In rural regions of India, *G. duodenalis* and *Cryptosporidium* spp. have been detected in humans, buffaloes, cattle, goats, sheep, dogs, and water sources (tube wells and lakes). These findings show preliminary evidence of the diversity of possible transmission routes and help to elucidate the distribution of these parasites in coexistence with humans and animals and their water sources (31).

CONTROL AND PREVENTION

To date, there is still no vaccine or drug that is effective in the treatment of ruminants with cryptosporidiosis, which makes the control of infection difficult. Therefore, the best strategy is to adopt management measures to reduce the spread of the disease in herds (126, 127).

The recommended methods for controlling *Cryptosporidium* spp. infection in ruminants are similar to those recommended for other protozoa and coccidia. Consequently, good breeding practices should be employed out, such as separating animals with diarrhea, cleaning the premises before the animals are introduced, removing and eliminating fecal content or wet garbage, cleaning feeders and drinking fountains, developing strategies to reduce humidity in facilities, and providing adequate supplies of colostrum to neonates (128, 129).

The survival time of oocysts can be reduced by freezing, desiccation, exposure to ultraviolet light, and variations in hydrogen potential (pH) and temperature (74). *Cryptosporidium* spp. oocysts are sensitive to ozone, at temperatures of 55°C for 30 s or 70°C for 5 s (130). Thus, viable solutions for disinfecting the environment consist of 10% formaldehyde for 18 h and 5 or 50% ammonia for 30 min (131–133).

In some studies, there has been a reduction in the excretion of *Cryptosporidium* spp. oocysts in cattle calves treated with azithromycin (134, 135). Other studies with intestinal microbiota found that fiber deprivation in the diet increases the susceptibility of mice to cryptosporidiosis, and there is a need for research to confirm this possibility in other animal models (136).

Such compounds such as paromomycin (9), albendazole (137, 138), and fenbendazole (9, 139) can be used for the treatment of giardiasis in cattle calves. The action of probiotics against *G. duodenalis* was primarily investigated *in vitro*. Probiotics that modulate the immune response have a beneficial effect on the composition of the intestinal microbiota and minimize parasite–host interactions (140, 141).

There is no vaccine available for the prevention and control of giardiasis in ruminants (142). Prophylactic measures are similar for the two protozoa and should include complete cleaning and disinfection of housing facilities using such products as ammonia, chlorine dioxide, hydrogen dioxide, and ozone. Additionally, maintaining a dry environment inside buildings may hinder the development of parasites (46, 143).

Minimizing the spread of *G. duodenalis* infections among ruminants is a considerable challenge (137). Thus, environmental disinfection associated with drug therapy is recommended (9, 137, 138). The daily removal of feces in the stable, pens, and surroundings is also important. Additionally, it is essential to ensure adequate intake of colostrum by newborns for the establishment of passive immunity (37). These measures need to be incorporated into the management of the herd with the main priority of adopting good health practices.

CONCLUSION

Based on recently published research, we demonstrate the global occurrence of *Cryptosporidium* and *G. duodenalis* in buffaloes from different geographic regions. Although these parasites possess biological differences, they are frequently discussed together because they share transmission pathways and cause diseases in the gastrointestinal tract. Water buffaloes can also become infected and excrete *Cryptosporidium* spp. oocysts and *G. duodenalis* cysts. In general, young buffaloes are more affected by these agents than are older animals. Infected hosts presenting with and without clinical manifestations can eliminate the infective forms of these protozoa in the environment. Cryptosporidiosis and giardiasis can be diagnosed by a wide variety of parasitological, serological, and molecular techniques. The application of molecular approaches for the identification of these two parasites has led to significant advances in knowledge regarding the epidemiology of these protozoans, with different species being characterized. The common occurrence of these parasites in both buffaloes and humans highlights the potential role of zoonotic transmission in the epidemiology of cryptosporidiosis and giardiasis. However, molecular methods are mostly restricted to research and specialized laboratories, and further research is warranted to determine the taxonomic status and host specificity of these genotypes found in water buffaloes. We recommend that measures need to be incorporated

into the management of the herd with the main priority of adopting good health practices and highlighting the importance of using molecular tools to identify species/genotypes for a better understanding of the epidemiology of these protozoa relevant in public health.

AUTHOR CONTRIBUTIONS

All authors have made substantial contributions to the conception of this work and critically reviewed for important intellectual content. We have also collectively approved the final version to be published and unanimously agreed with all aspects of this work, ensuring that issues related to the accuracy and integrity of any part of this work are properly investigated and resolved.

FUNDING

This review was financed by the São Paulo State Research Support Foundation (acronym in Portuguese, FAPESP), process number 2011/525423 and CAPES PROEX 0549/2019. JG is recipient of CNPq fellowship.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 05 November 2020
doi: 10.3389/fvets.2020.568249

Polymorphisms in *TLR4* Gene Associated With Somatic Cell Score in Water Buffaloes (*Bubalus bubalis*)

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OPEN ACCESS

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: 31 May 2020
Accepted: 14 October 2020
Published: 05 November 2020

Citation:
Roldan-Montes V, Cardoso DF, Hurtado-Lugo NA, do Nascimento AV, Santos DJdA, Scalez DCB, de Freitas AC, Herrera AC, Albuquerque LG, de Camargo GMF and Tonhati H (2020) Polymorphisms in TLR4 Gene Associated With Somatic Cell Score in Water Buffaloes (Bubalus bubalis). Front. Vet. Sci. 7:568249. doi: 10.3389/fvets.2020.568249

Considering the importance of the diseases affecting the productive performance of animals in the dairy industry worldwide, it is necessary to implement tools that help to control and limit the occurrence of such diseases. As the increased somatic cell counts (SCC) are a direct expression of the inflammatory process, they are candidates to become the usual parameter for assessing udder health regarding milk quality and for monitoring mastitis incidences. Toll-Like Receptors are membrane proteins that play a key role in immunity, recognizing pathogens and, subsequently, activating immune responses. The present study was conducted to identify single nucleotide polymorphisms in the *TLR4* gene of buffaloes and to analyze its associations with somatic cell counts. DNA samples of 120 Murrah buffaloes were used. The whole coding region of the *TLR4* gene was amplified by polymerase chain reaction reactions and sequenced for polymorphism scanning. A total of 13 polymorphisms were identified for the sequenced regions of the *TLR4*, most of which are in the coding region. The association with the somatic cell score was highly significant ($p < 0.001$) for all identified polymorphisms of *TLR4* gene (g.54621T>A, g.54429G>T, g.54407T>A, g.46616C>A, g.46613T>G, g.46612A>G, g.46611C>A, g.46609T>G, g.46541C>G, g.46526C>A, g.46516T>C, g.46376C>T, g.46372T>C). Therefore, it is suggested that the markers of the *TLR4* gene can be used as molecular markers for mastitis resistance in buffaloes, due to their association with somatic cell counts.

Keywords: milk yield, SNP, stop codon, udder health, molecular marker analysis, mastitis

INTRODUCTION

The water buffalo (*Bubalus bubalis*) is a worldwide species used as a source of draft power, milk, and meat (1). The milk production represents an economically interesting activity, especially because of appreciated milk derivatives, such as "mozzarella," and the remarkably yield efficiency manufacturing dairy products, due to buffalo's milk physical-chemical properties with high levels

of protein, fat, and minerals (2–5). This species can be affected by health problems similar to those presented by cattle, among them the occurrence of mastitis (6), which consists of inflammatory reactions of the mammary gland triggered by the invasion of pathogens or traumatic events (7). The most frequently isolated pathogens in milk samples of buffaloes with mastitis are gram-positive bacteria, such as *Staphylococcus spp*. *Streptococcus spp*. (6, 8), and which causes mostly subclinical mastitis (9). However, low incidence of infection by Gram-negative bacteria, such as *Escherichia coli* and *Klebsiella pneumoniae*, has also been reported in the species (10, 11). Further than changes in milk composition, mastitis' major impacts on the dairy industry include the deterioration of animal comfort and welfare, the reduced performance of herds, increased therapeutic costs, and involuntary culling of cows early in lactation (12). Therefore, it is of paramount importance the development of strategies to reduce the incidence of this disease, such as the implementation of improved management practices and enhance of mastitis resistance in dairy herds through selection.

Subclinical mastitis, the most common inflammation of mammary tissue, is asymptomatic. Therefore, its diagnosis and onset of treatment rely largely on indirect indicators of inflammation, like the somatic cell count (SCC) test (13). The presence of somatic cells in milk is a normal physiological phenomenon even in healthy animals. Leukocytes and epithelial cells are constantly circulating in the mammary gland to guarantee its surveillance and protection against infection (14). However, increased SCC in the milk indicates the recruitment of the first line of cellular defense, particularly neutrophils, from blood to the mammary gland in response to long term intramammary inflammation caused by gram-positive bacteria (15). Analogously to bovine, the SCC limit of 200,000 cells/ml is usually adopted to define subclinical mastitis in buffaloes (6, 8, 14, 16), whereas values ranging between 11,000 and 171,000 cells/ml have been reported in the milk of healthy cows (11, 17). According to Salvador et al. (18) and Cerón-Muñoz et al. (17), the prevalence of subclinical mastitis in the Philippine and Brazilian water buffaloes herds was 42.8 and 3.2%, respectively.

Beyond being the most indicator of subclinical mastitis, SCC is widely applied as a selection criterion for enhanced resistance to mastitis, either subclinical or clinical (19). Although there is some controversy regarding the relation of low SCC and clinical mastitis resistance (13), studies in sheep and cattle have demonstrated that animals with higher SCC are most prone to mastitis (20, 21), and in agreement with these studies, the genetic correlation between SCC and clinical mastitis varying from moderate to high (0.59–0.77) have been reported in dairy cattle (22–24). In addition to its high correlation with clinical mastitis, the SCC represents an objective measure feasible of being periodically recorded in large populations with higher estimates of heritability than for clinical mastitis itself (25).

The comprehension of genomic regions and genes underlying the genetic variation in SCC of buffaloes might be useful for the assessment of genetic variability of mastitis resistance in the species, and it may assist the early selection of more robust animals. In addition, it is a pivotal first step for a better understanding of biological mechanisms behind the mastitis resistance and interpretation of the relation between clinical and subclinical mastitis, which are mostly associated with the activation of the innate and adaptive immune system, respectively (15). Toll-like receptor 4 (*TLR4*) is a pattern-recognition receptor that plays key roles stimulating the immune system by binding to pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide, an outer membrane component of gram-negative bacteria, as well as lipoteichoic acid present in the wall of some of the gram-positive bacteria (26, 27). Despite *TLR4* main importance to induce an innate immune response, it was an overexpressed gene in the mammary gland of bovine cows (28), and in the milk of buffaloes (29), infected exclusively by gram-positive bacteria. Therefore, *TLR4* corresponds to an interesting candidate gene for SCC, due its dual specificity to both gram-negative and –positive bacteria, hence its potential roles linking adaptive and innate immune systems (30). Moreover, Mesquita et al. (31) found a significant association between the polymorphisms of the *TLR4* and the SCC in Holstein cows, suggesting its potential to improve udder health through marker-assisted selection.

The present study aimed to verify the existence of polymorphisms in the coding region of the *TLR4* gene in a Brazilian buffalo population and verify its potential as molecular markers for infection resistance in the species, through assessing the association between *TLR4* polymorphisms and SCC in buffalo milk. In addition, we contrast the predicted protein structure of the *TLR4* gene of buffaloes and cattle.

MATERIALS AND METHODS

Animals

The protocol for the present study was based on guidelines of the National Council for Animal Experimentation Control (CONCEA) and approved by the Committee on Ethics in the use of animals (CEUA) (protocol number 014624/17).

The phenotypic data and the biological material used in this study were provided by the Tapuio farm located in Taipu-RN, Brazil, which is part of the buffalo milk-recording program maintained by the Animal Science Department of the São Paulo State University (Unesp). The farms were free of brucellosis, tuberculosis, and leucosis and strictly follow the Brazilian vaccination calendar. The current herd has approximately 688 buffalo lactating dams, with average milk production of 2,042.11 ± 681.76 kg in up to 270 days of lactation. A total of 120 first-lactation cows that had milk sampled every other month during 2016 for determining the SCC were chosen for the genotyping process. The choice of the 120 cows considered a sampling that represented the genetic diversity of the herd and composed contemporary groups that contained at least three animals. The average of SCC in the milk analyses was 36,100 ± 586,000 cells/ml (ranging from 10,000 to 3,258,000 cells/ml). None of the animals presented clinical mastitis, only subclinical mastitis according to values of SCC exceeding 200,000 cells/ml. The large variation in the SCC makes the studied population a valuable source to initial evaluation of the association of the candidate gene *TLR4* and the udder-health indicator trait SCC.

Genomic DNA from 120 Murrah animals was extracted from tail hair follicles using the commercial extraction kit Macherey-Nagel NucleoSpin® Tissue (Düren, Germany).

Primer Designs and PCR Reactions

The primer pairs used to amplify the coding region of the *TLR4* gene (**Table 1**) were designed using the Primer3 tool (http://bioinfo.ut.ee/primer3-0.4.0/), based on the *Bos taurus* sequence (GenBank accession number: AC_000165.1). The *TLR4* gene consists of 3 exons. Exons 1 and 3 include coding regions and the non-coding regions 5'UTR and 3'UTR, respectively. One pair of primers was designed for exons 1 and 2 each, whereas three further pairs of primers were designed for exon 3.

The amplification of coding regions of the *TLR4* gene was performed by polymerase chain reaction (PCR) assays, which were conducted in Bio-rad S1000 thermal cyclers (Bio-Rad, Hercules, CA, USA) in a final volume of 15 μL, consisting of 100 ng of genomic DNA, 15 pM of each primer and the GoTaq Colorless MasterMix kit (Promega, Madison, WI, USA). The thermal profile was expressed by initial denaturation at 95°C for 5 min, followed by 34 cycles of denaturation at 95°C for 30 s; annealing at the melting temperature of each primer (**Table 1**) pair for 30 s; and extension at 72°C for 30 s. A final extension at 72°C for 5 min was performed. Further, 2 μL of PCR products were visualized by electrophoresis on 2% agarose gels stained with the GelRed system (Biotium, Inc., Hayward, CA, USA).

Sequencing of Amplified Fragments

Each amplicon of the 120 samples was purified following the protocol recommended by the Wizard SV Gel and PCR Clean-Up System (Promega) kit. Then, they were sequenced from both primers (Forward and Reverse) by the dideoxynucleotide chain termination technique (ddNTPs) using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) in an automatic ABI 3730 XL sequencer (Applied Biosystems). Then, the obtained DNA sequences were analyzed for identifying the polymorphisms using the CodonCode Aligner program. The structure of the *TLR4* buffalo protein was predicted by the Modeler software (32) while the three-dimensional structures of the proteins were generated by the UCSF Chimera (33).

Allele and Genotype Frequencies

The allele and genotype frequencies of the polymorphisms detected in the *TLR4* gene were estimated for the population by counting. The Hardy-Weinberg equilibrium was verified for each polymorphic site by the chi-square test (χ^2) ($p < 0.05$). The linkage disequilibrium between the pairs of markers was estimated from the r^2 statistics (34) using the Plink software (35).

Association Analysis

The SCC evaluated in the association study refers to the first lactation records of 120 dairy buffaloes. The association analyses were performed by a mixed model using univariate analyzes in the Proc Mixed procedure of the SAS/STAT 9.3 statistical program (SAS Institute, Inc., Cary, NC, USA). The following statistical model was applied:

$$y_{ijkl} = \mu + CG_i + b_1 IV_j + b_2 IV_j^2 + M_l + T_k + e_{ijkl}$$

where, y_{ijkl} is the SCC determined for the ijklth animal; μ is the average of the SCC in the population; CG_i is the fixed effect of ith contemporary group (year and season of birth); IV_j is the age of the jth dam at birth, considered as a linear and quadratic (co)variable in the model; $M_l$is the fixed effect of the lth genotype for each polymorphism detected in the *TLR4* gene; T_k is the random effect of the sire, and e_{ijkl} is the residual random effect associated with the observation y_{ijkl}.

Since the SCC data do not have a normal distribution, the generalized linear mixed model was performed assuming Poisson distribution for the trait. To evaluate the association of the marker with the studied trait, a significance threshold for the *P*-value was calculated by Bonferroni correction ($\alpha = 0.05/N_{polymorphisms}$). The additive and the dominance

TABLE 1 | Identifying the primers, the *TLR4* gene regions where the primers anneal, annealing temperature, and size of the amplified fragment.

Primers ID	Primers*	T melting (°C)	Fragment size (pb)
1	F: 5′ ACAGGGAGAAGACAGCCAT 3′ R: 5′ CAAATGAACCTAACCAG 3′	54.4°C	728
2	F: 5′ AAATGAAGGGATAAGTG 3′ R: 5′ CTGAAGAAGGGAGAT 3′	43.3°C	535
3A*	F: 5′ TTTCATTTTGGTTTCCTAT 3′ R: 5′ TTATATCTTTGTTGTCTG 3′	49°C	894
3B*	F: 5′ CAAGGGTTGCTGTTCTCACA 3′ R: 5′ GGAAACTCTGATGTTC 3′	48.9°C	758
3C*	F: 5′ AAGGAGCAAGAACTACA 3′ R: 5′ AAAGAAGCACATCAGGGGA 3′	52.5°C	877

*F, forward; R, reverse; 3A, 3B, 3C are the primers used in exon 3.

TABLE 2 | SNPs and amino acid changes in the *TLR4* gene.

Exon	SNP*	Amino acid change
1	g.54621T>A	–
1	g.54429G>T	Thr/Met
1	g.54407T>A	Arg/Ser
3	g.46616C>A	Cys/Tyr
3	g.46613T>G	Tyr/Cys
3	g.46612A>G	Tyr/Stp
3	g.46611C>A	Glu/Lys
3	g.46609T>G	Glu/Glu
3	g.46541C>G	Gly/Asp
3	g.46526C>A	Gly/Asp
3	g.46516T>C	Lys/Asn
3	g.46376C>T	Arg/Ile
3	g.46372T>C	Leu/Phe

*Sequence-based marker positioning >NW_005784801.1.

effects of the significant SNPs were tested after analyzing the associations between the SNPs and SCC. These analyses were performed by orthogonal contrasts using the PROC GLM in SAS (SAS 9.2, SAS Institute, Cary, NC, USA).

The probabilities of haplotypes were constructed in the Haploview software to analyze and visualize linkage disequilibrium patterns (36), using the block system reported by Gabriel et al. (37) where the region is segmented according to the LD.

RESULTS AND DISCUSSION

A total of 13 SNPs were identified in the studied population (**Table 2**), three in exon 1 and 10 in exon 3 of the *TLR4* gene. The SNP g.54621T>A of exon 1 was the only one located in a non-coding region (5'UTR), whereas the SNP g.46616C>A|T, of the same exon, was the only polymorphism with more than two alternative alleles. The three alleles of this *locus* lead to three different amino acids in the coded protein, increasing the protein variability level. Moreover, some of the identified polymorphisms were located in the same codon (e.g., g.46611C>A, g.46612A>G and g.46613T>G), which increases the number of possible nucleotide combination in a codon and also contributes to the protein variability. Almost all SNPs found in the *TLR4* gene have non-synonymous conservative amino acid substitutions in the protein. The fact that the *TLR4* gene is a cell-surface pattern-recognition receptor important to recognition of a broad of PAMPs, somehow justifies the amount of SNPs and the variability of predicted protein isoforms detected in this sample. White et al. (38) also reported a relative abundance of polymorphism ($N = 28$) in the coding region *TLR4* gene in a sample involving 11 different cattle breeds. Similarly, a large amount of polymorphisms has been shown in the coding region of *TLR4* in human populations ($N > 50$) (39). Consulting the Ensembl variation resource (40), we observed that 224, 61, and 36 variations have been described in the coding region of *TLR4* in cattle (ENSBTAT00000008190.3), sheep (ENSOART00000006304.1), and goat (ENSCHIT00000027327.1), respectively.

TABLE 3 | Estimates of linkage disequilibrium (r^2) of the *TLR4* SNPs.

SNP	g.54429 G>T	g.54407 T>A	g.46616 C>A	g.46613 T>G	g.46612 A>G	g.46611 C>A	g.46609 T>G	g.46541 C>G	g.46526 C>A	g.46516 T>C	g.46376 C>T	g.46372 T>C
g.54621T>A	0.929	0.946	0.008	0.011	0.002	0.012	0.255	0.014	0.023	0.032	0.003	0.008
g.54429G>T		0.982	0.017	0.022	0.004	0.016	0.030	0.021	0.031	0.026	0.010	0.010
g.54407T>A			0.017	0.023	0.004	0.019	0.028	0.020	0.030	0.027	0.009	0.009
g.46616C>A				0.155	0.726	0.737	0.648	0.800	0.852	0.246	0.774	0.678
g.46613T>G					0.071	0.171	0.197	0.165	0.144	0.089	0.142	0.176
g.46612A>G						0.594	0.588	0.763	0.758	0.311	0.671	0.713
g.46611C>A							0.789	0.748	0.764	0.243	0.706	0.651
g.46609T>G								0.759	0.770	0.277	0.648	0.708
g.46541C>G									0.916	0.305	0.828	0.849
g.46526C>A										0.302	0.806	0.828
g.46516T>C											0.251	0.322
g.46376C>T												0.796

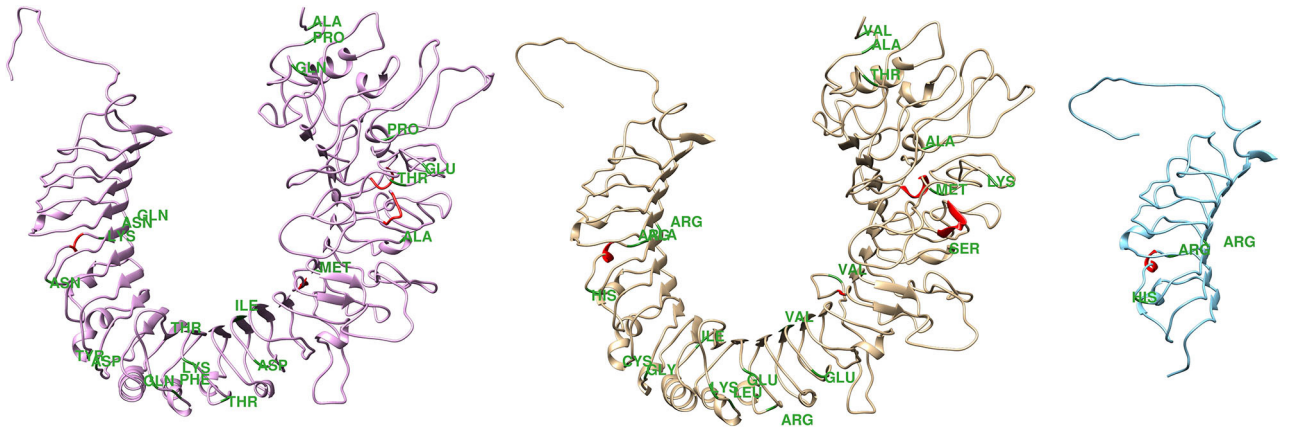

FIGURE 1 | Theoretical three-dimensional model for bovine and buffalo proteins. From right to left are represented the predicted structure of the bovine protein, buffalo protein in the absence and presence of premature stop codon. * Green indicates amino acid exchanges.

A noteworthy variation in the population refers to the possible combination of the alleles A and T of the SNPs g.46612A>G and g.46613T>G, respectively. This allelic combination corresponds to the establishment of a premature stop codon, which causes the interruption of the protein synthesis with 191 amino acids instead of the 841 expected under normal synthesis (41, 42). Surprisingly, 3 of the healthy cows presented two copies of haplotype AT (i.e., AA in SNP g.46612A>G and TT in the g.46613T>G), which corresponds to two copies of the premature stop codon and absence of functional *TLR4* protein. Despite the direct influence of *TLR4* in the immune system, this non-sense mutation did not represent a recessive lethal combination, although the truncated protein did not completely encompass the predicted signaling domain of its wild type *TLR4* (43). It is questionable that these animals have survived up to adult age without being naturally exposed to gram-negative bacterial infections. Therefore, our findings raise a point to be further investigated about the biological mechanisms compensating the *TLR4* absence in these buffaloes samples when it becomes essential. It is noteworthy that the two adjacent SNPs g.46612A>G and g.46613T>G presented the lowest linkage disequilibrium measured by r^2 between pairwise of SNPs in exon 3 ($r^2 = 0.07$, **Table 3**), which might represent purifying selection frequently acting on the stop codon determinant haplotypes, AT.

Beyond the SNPs in the buffalo species, the sequencing of the amplicons revealed 45 punctual differences in coding regions across buffaloes and cattle (**Supplementary Table 1**), 20 of them resulting in amino acids alterations. Some of the across species missense variation were non-conservative, suggesting that structural and functional differences exist between the species (44). **Figure 1** shows the three-dimensional protein structure predicted for each bovine and buffalo species, as well as the predicted structure in the presence of a premature stop codon. It has been postulated that buffaloes are less susceptible to bacterial infection than cattle, due to some anatomic attributes, such as their longer narrow teat compared to bovine, which prevents pathogens invasion (6). The structural difference in proteins encoded by *TLR4* genes of the two species might represent an additional physiological mechanism empowering this difference in mastitis resistance.

The allele and genotype frequencies of each SNP are shown in **Table 4**. Most SNPs had minor allele frequency (MAF) higher than 0.10, which is favorable to performing association analyses since low MAF could affect the prediction of SNP effects (45). Most of the SNP frequencies adhered to the expected value of

TABLE 4 | Allele and genotype frequencies and Hardy-Weinberg equilibrium of the polymorphisms found in the *TLR4* gene.

SNP	Allele frequency		Genotype frequency			H-W equilibrium
	T	**A**	**TT**	**TA**	**AA**	χ^2
g.54621T>A	0.2864	0.7146	0.0971	0.3786	0.5243	0.4715
	G	**T**	**TT**	**GT**	**GG**	
g.54429G>T	0.7048	0.2952	0.5192	0.3846	0.1058	0.3585
	A	**T**	**AA**	**AT**	**TT**	
g.54407T>A	0.7165	0.2835	0.1134	0.3402	0.5464	0.1316
	A	**C**	**AA**	**AC**	**CC**	
g.46616C>A	0.5818	0.4182	0.3727	0.4182	0.2091	0.1692
	T	**G**	**TT**	**TG**	**GG**	
g.46613T>G	0.9204	0.0797	0.8496	0.1416	0.0088	0.5203
	A	**G**	**AA**	**AG**	**GG**	
g.46612A>G	0.4789	0.5211	0.3474	0.2632	0.3895	4.067*
	C	**A**	**CC**	**CA**	**AA**	
g.46611C>A	0.65	0.35	0.50	0.30	0.20	0.0006
	T	**G**	**TT**	**TG**	**GG**	
g.46609T>G	0.3785	0.6215	0.4299	0.3832	0.1869	0.0639
	C	**G**	**CC**	**CG**	**GG**	
g.46541C>G	0.5841	0.4159	0.3540	0.4602	0.1858	0.5656
	C	**A**	**CC**	**AC**	**AA**	
g.46526C>A	0.4306	0.5694	0.2222	0.4167	0.3611	0.1195
	T	**C**	**TT**	**TC**	**CC**	
g.46516T>C	0.6712	0.3288	0.26	0.37	0.37	3.382
	C	**T**	**CC**	**CT**	**TT**	
g.46376C>T	0.5585	0.4415	0.3723	0.3723	0.2553	0.0209
	T	**C**	**TT**	**CT**	**CC**	
g.46372T>C	0.4307	0.5693	0.2277	0.4059	0.3663	0.1034

**SNP not in Hardy-Weinberg equilibrium.*

the Hardy-Weinberg Equilibrium verified by the chi-square test (χ^2) ($P < 0.05$). However, two of them were not in equilibrium, including the SNP g.46612A>G that is related to the premature stop codon. Another indication that purifying selection, or other of the factors that affect Hardy-Weinberg Equilibrium, might be acting over this particular *locus*.

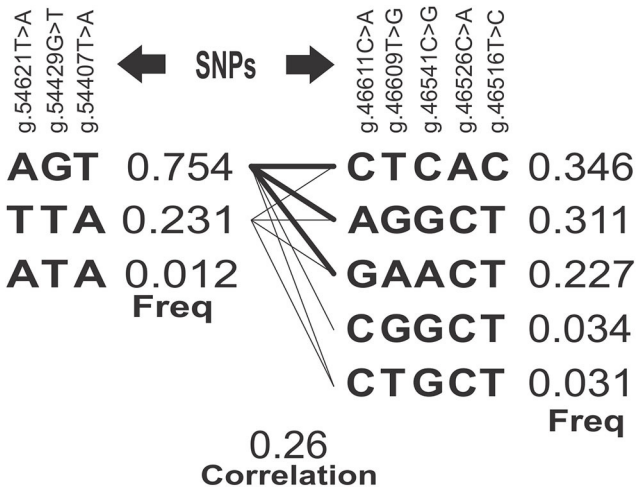

FIGURE 2 | Blocks of haplotypes estimated correlations and frequencies. SNPs, Single Nucleotide Polymorphism; Freq, Frequency estimated for each haplotype.

SNPs with higher r^2 tend to segregate together because they are closer. The LD of the g.54621T>A, g.54429G>T, and g.54407T>A SNPs (**Table 3**) suggested very high concordance between the genotypes of three *loci* (0.929, 0.946, 0.982), indicating that their alleles segregate together. Therefore, any of the three SNPs might equally capture the proportion of the genetic variation of the SCC trait, which is attributed to a causative mutation close/among them or to the block. Accordingly, five SNPs of the exon 3 (g.46611C>A, g.46609T>G, g.46541C>G, g.46526C>A, and g.46516T>C) also represented a block in high LD. The correlation between the blocks (0.26) and the estimated frequencies for each of the haplotypes is shown in **Figure 2**, being ACA and GAAAC the most common haplotypes in exons 1 and 3 with estimated frequencies of 0.75 and 0.34, respectively.

Adjusted means of SCC were compared according to the genotype classes of each SNP, and significant differences ($P < 0.0001$) were observed in all *loci* (**Table 5**). A significant effect of allele substitution was observed for all SNPs, indicating phenotypical differences associated with the number of copies of favorable alleles in each SNP, even if some of them are not the causative mutations. As selection for SCC would target the lower values, due to its association with great mastitis resistance, the alleles related to the reduction in the averages would be

TABLE 5 | Adjusted means and standard errors of somatic cell counts (SCC, 1,000/ml) per genotypes, substitution allele, and dominance effect.

SNP	Averages of genotypes*			Allele effect	*P*	Dominance effect	*P*
	TT	**AT**	**AA**				
g.54621T>A	4.81 ± 0.34[b]	5.65 ± 0.34[a]	5.56 ± 0.34[a]	0.44 ± 0.01	<0.0001	0.01 ± 0.13	0.98
	GG	**GT**	**TT**				
g.54429G>T	4.86 ± 0.31[c]	5.47 ± 0.31[b]	5.63 ± 0.31[a]	0.42 ± 0.01	<0.0001	0.02 ± 0.13	0.86
	TT	**AT**	**AA**				
g.54407T>A	5.73 ± 0.37[a]	5.74 ± 0.37[a]	4.76 ± 0.37[b]	0.51 ± 0.01	<0.0001	−0.03 ± 0.14	0.83
	CC	**AC**	**AA**				
g.46616C>A	5.73 ± 0.30[a]	5.48 ± 0.30[b]	4.86 ± 0.30[c]	−0.41 ± 0.01	<0.0001	−0.28 ± 0.01	<0.0001
	TT	**TG**	**GG**				
g.46613T>G	5.27 ± 0.26[b]	5.80 ± 0.26[a]	6.13 ± 0.99[a]	0.56 ± 0.02	<0.0001	0.04 ± 0.17	0.82
	AA	**AG**	**GG**				
g.46612A>G	5.92 ± 0.33[a]	4.61 ± 0.33[b]	4.33 ± 0.33[c]	−0.53 ± 0.01	<0.0001	−0.12 ± 0.15	0.43
	CC	**CA**	**AA**				
g.46611C>A	5.00 ± 0.28[a]	5.38 ± 0.28[b]	5.70 ± 0.28[c]	0.29 ± 0.01	<0.0001	0.08 ± 0.01	<0.0001
	TT	**TG**	**GG**				
g.46609T>G	4.88 ± 0.29[c]	5.43 ± 0.29[b]	6.18 ± 0.29[a]	0.49 ± 0.01	<0.0001	0.05 ± 0.12	0.66
	CC	**CG**	**GG**				
g.46541C>G	4.76 ± 0.31[c]	5.46 ± 0.31[b]	5.86 ± 0.31[a]	0.47 ± 0.01	<0.0001	0.20 ± 0.01	<0.0001
	CC	**CA**	**AA**				
g.46526C>A	6.06 ± 0.36[a]	5.37 ± 0.36[b]	4.96 ± 0.36[c]	−0.24 ± 0.01	<0.0001	−0.07 ± 0.01	< 0.0001
	TT	**AT**	**CC**				
g.46516T>C	5.79 ± 0.25[a]	4.68 ± 0.25[b]	4.59 ± 0.25[c]	−0.57 ± 0.01	< 0.0001	−0.11 ± 0.16	0.48
	CC	**CT**	**TT**				
g.46376C>T	5.03 ± 0.2[b]	4.84 ± 0.2[c]	5.53 ± 0.2[a]	−0.03 ± 0.01	<0.0001	−0.12 ± 0.13	0.38
	TT	**TC**	**CC**				
g.46372T>C	4.72 ± 0.28[c]	5.21 ± 0.28[b]	5.85 ± 0.28[a]	0.21 ± 0.01	<0.0001	0.07 ± 0.13	0.58

**a, b, c different letters in the same row indicate significant differences (p < 0.006) by the Bonferroni test. p, p-value.*

favorable, e.g., the allele T of SNP g.54621T>A and the allele A of the SNP g.46616C>A. Roughly, for SNP g.54621T>A, where the allele substitution was modeled as replace of alleles Ts by As ("T>A" representing TT > TA >AA), the SNP effect represents the regression coefficient of SCC on the genotypes TT, TA, AA, hence the substitution effect of A allele (**Table 5**). Additionally, some SNPs showed a significant dominance effect, including g.46616C>A, g.46611C>A, g.46541C>G, and g.46526C>A.

To the best of our knowledge, it is the first study of the effect of SNP in the *TRL4* gene on the SCC in buffaloes, demonstrating that *TRL4* polymorphisms are associated with the SCC concentration in the milk of healthy animals. Only a few studies regarding candidate genes for economically important traits are available in buffaloes (46–49). For mastitis resistance, they are even scarcer (50). Alfano et al. (51) reported significant results for the tuberculosis resistance of SNPs in the *TRL4* gene of dairy buffaloes. The SNP (672A>C) described by these authors is the same reported here, as SNP g.46516T>C. It evidences the importance of gene polymorphisms in the immune system of ruminants. In cattle, *TLR4* polymorphisms have been associated with SCC (31, 52). Thus, despite the large number of non-synonymous divergences between the amino acids in the *TLR4* of buffaloes and bovine, the roles of the gene in the immune response of the mammary gland is preserved in both species.

DNA polymorphism can be used to assist the selection for a specific trait when it is associated with such traits. Specific markers can also be used to avoid some congenital recessive defects of genetic origin that affects cattle, such as bovine leukocyte adhesion deficiency (BLAD, 40). Considering the impact of mastitis in milk production, the usefulness of SCC as an indicator of mastitis resistance, and the results found in this study, there is evidence that the *TLR4* gene can be used for marker-assisted selection. We need to emphasize that even finding promising results, this is a preliminary study with a small population. Further studies involving a larger sample, more generations, and animals with clinical mastitis are encouraged to validate these results. Additionally, the milk cell profile of cows that were homozygous for the premature stop codon, as well as the animal reaction face bacterial challenge are yet to be adequately assessed. The intriguing point behind the stop codon detected in the current study is the well-known importance of *TLR4* for the immune system, especially for immune responses to gram-negative bacteria. Other non-lethal premature stop codons have been reported for other genes in the literature. For instance, a non-sense mutation was also reported in buffaloes *JY-1*, which encodes an oocyte-specific protein. Additionally, a premature stop codon created a novel favorable allele for muscle development in the *GDF8* of cattle (53).

The *TLR4* buffalo gene shows a different coding region in comparison to the cattle sequence with many non-synonymous polymorphisms. *TLR4* gene is highly polymorphic in buffaloes, especially for exon 3. The non-synonymous polymorphisms herein presented show potential as molecular markers for SCC in buffaloes and likely for resistance to clinical mastitis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, NW_005784801.1.

ETHICS STATEMENT

The animal study was reviewed and approved by National Council for Animal Experimentation Control (CONCEA). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

VR-M, NH-L, GdC, and HT planned the study. VR-M, DC, AdN, and AdF performed the molecular. NH-L, AH, DS, and DSa performed the curation of data and statistical analyses. LA and DC supported the data analysis and contributed to the interpretation of the results. VR-M composed the original draft. All authors read and approved the current draft.

ACKNOWLEDGMENTS

The authors thank the grant #2014/25309-7, São Paulo Research Foundation (FAPESP) provided as financial support to conduct this research project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.568249/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 10 November 2020
doi: 10.3389/fvets.2020.552413

Comparative Analysis Using Pulsed-Field Gel Electrophoresis Highlights a Potential Transmission of *Salmonella* Between Asymptomatic Buffaloes and Pigs in a Single Farm

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OPEN ACCESS

Edited by:
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Reviewed by:
Alexander Van Parys, Ghent University, Belgium
Friederike Hilbert, University of Veterinary Medicine Vienna, Austria

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *16 April 2020*
Accepted: *21 October 2020*
Published: *10 November 2020*

Citation:
Santana AM, Silva DG, Maluta RP, Pizauro LJL, Simplício KMMG, Santana CH, Rodrigues SAD, Rodrigues DP and Fagliari JJ (2020) Comparative Analysis Using Pulsed-Field Gel Electrophoresis Highlights a Potential Transmission of Salmonella Between Asymptomatic Buffaloes and Pigs in a Single Farm. Front. Vet. Sci. 7:552413. doi: 10.3389/fvets.2020.552413

Buffaloes and pigs play an important epidemiological roll in the *Salmonella* infection cycle, and asymptomatic animals can act as key component in the dissemination of the disease by horizontal, vertical, and cross-species transmission. Our study aimed and was able to confirm evidences of a cross-species transmission of *Salmonella* Agona between asymptomatic buffaloes and pigs. Also, we described *Salmonella* infection within the pig production phases, involving serotypes Agona, Senftenberg and Schwarzengrund. Rectal samples were collected from Jafarabadi buffaloes ($n = 25$) and Piau pigs ($n = 32$), located on a single farm. *Salmonella* Agona was isolated from lactating buffaloes, gilts, pregnant sows, and weaned pigs, *Salmonella* Schwarzengrund from lactating sows and *Salmonella* Senftenberg from gilts, pregnant sows, lactating sows, and weaned pigs. Pulsed-field Gel Electrophoresis protocol (PFGE) was performed and revealed four different profiles. Profile 1 (*Salmonella* Agona), isolated from a pregnant sow, a gilt and two lactating buffaloes, revealed a indistinguishable PFGE pattern, confirming evidences of potential cross-species transmission. Profile 2 (*Salmonella* Agona), 3 (*Salmonella* Senftenberg), and 4 (*Salmonella* Schwarzengrund), isolated from pigs, revealed important indistinguishable PFGE patterns, evidencing *Salmonella* infection within the pig production phases. Considering the epidemiological relevance of buffaloes

and pigs in the cycle of *Salmonella* infection, confirmation of a potential cross-species transmission of *Salmonella* Agona and potential *Salmonella* infection within the pig production phases highlights the importance of the correct establishment of preventive health strategies in farms, in special the importance of avoiding contact between buffaloes and pigs, since cross-species transmission can occur, increasing the risk of spreading the disease.

Keywords: *Bubalus bubalis*, epidemiology, feces, Jafarabadi, porcine, *Salmonella* Agona, serotypes, swines

INTRODUCTION

Salmonella is one of the most important pathogens in livestock animals, and is a matter of concern, as it may be responsible for great economic losses within the herd, as well as being a zoonotic agent linked to foodborne illness and therefore a major public health concern worldwide (1). In buffaloes, *Salmonella* is also responsible for economic losses (2), and several serotypes have been reported worldwide (2–8), isolated in feces (2, 3, 5, 6), raw meat (4, 8) and milk (7). Although reports show that salmonellosis in buffalo-calves is a widespread disease characterized by gastrointestinal lesions, diarrhea, hyperthermia, and dehydration (2, 9, 10), the major source of infection in the herd are asymptomatic adult animals shedding the bacteria through the feces (2), which is a great concern since these animals can act as reservoirs and be a great risk of transmission to humans and other animal species (3).

In pigs, *Salmonella* is also a matter of concern, being that a great diversity of serotypes have been reported, isolated in pig feces, lymph nodes/tonsils, and subproducts (1, 8, 11–18). *S.* Choleraesuis, serotype adapted to pigs, often cause the septicemic form of the disease, while *S.* Typhimurium is responsible for the enterocolitic form of the disease (19, 20). On the other hand, several *Salmonella* serotypes have been linked to asymptomatic pig carriers, being the main risk factor for the contamination of the final product during harvest and thereby presenting a food safety concern (21). Therefore, asymptomatic pigs represent a constant risk of *Salmonella* transmission to humans and other animal species (1, 22). Pork meat has been considered one of the major food products of animal origin responsible for *Salmonella* transmission to humans in diverse countries, including industrialized ones (22–24), being responsible for outbreaks in humans, as described in the literature for *S.* Agona (25), *S.* Senftenberg (26), and *S.* Schwarzengrund (27).

With this study, we aimed and we were able to show evidences of a potential transmission of *Salmonella* between asymptomatic buffaloes and pigs. Also, we were able to describe a *Salmonella* infection within the pig production phases. Therefore, these evidences highlight the importance of establishing preventive health strategies, among them avoiding contact between buffaloes and pigs, since *Salmonella* is a matter of concern for these two animal species, as well as an important public health problem worldwide.

MATERIALS AND METHODS

The Population Studied and Number of Samples Collected

This research was approved by the Ethics Committee on Animal Use of "Faculdade de Ciências Agrárias e Veterinárias, UNESP" (Protocol no 010885-08).

Samples were collected from Jafarabadi lactating buffaloes and five categories of Piau pigs within the production chain inside the farm (pregnant sows, lactating sows, gilts, boars, and weaned pigs). These animals were located on a single farm, in São Paulo State, Brazil. The lactating buffaloes were raised in a semi-intensive system, with a diet based on roughage and chopped sugar cane, supplemented with protein concentrate. The animals were housed during the night and released to graze in paddocks during the day, after the morning milking was performed. The pigs were raised in a semi-intensive system, with a diet based on a ration containing corn and soybean meal, supplemented with mineral core. The pigs were housed during the night and released to graze in paddocks during the day. Although the categories of pigs should be allocated into different structures, appropriate to each stage of the production chain, this did not occur on this farm. Thus, animals from all stages shared the same installation, in an inadequate breeding system where all animals had direct contact. Also, buffaloes and pigs, although housed in separate facilities during the night, were released in the same paddocks during the day, and therefore had direct contact. While in the paddocks, pigs could not access buffaloes feeders and water system because they were placed to high for the pigs. Therefore, the pigs had access to feeders and water through an adapted creep feeding system located inside the paddocks, where the buffaloes had no access.

A total of 25 rectal swab samples from Jafarabadi lactating buffaloes and 32 rectal swab samples from Piau pigs were collected for microbiological isolation of *Salmonella*. Rectal swab samples were collected from different categories of pigs within the production chain inside the farm: pregnant sows ($n = 5$), lactating sows ($n = 5$), gilts ($n = 8$), boars ($n = 3$), and weaned pigs ($n = 11$). Samples were collected at only one timepoint and on the same day.

Sampling and Initial Microbiological Procedures

To evaluate the presence of *Salmonella*, three fecal samples were collected from the rectum of each animal, with a cotton swab,

that was immediately transferred into tubes containing 10 ml of the selective enrichment broths selenite cystine (SC), Muller-Kauffmann tetrathionate (MKT) and Rappaport-Vassiliadis (RV). All samples were then transported to the laboratory in thermal boxes containing ice (time range from 2 to 3 h), where all microbiological procedures were performed.

At the "Research Support Laboratory of the Department of Veterinary Clinic and Surgery, FCAV, UNESP, Jaboticabal Campus, Brazil," selective enrichment broths were incubated at 37°C for 24 h. After incubation, the broths (SC, TMK, and RV) were seeded on plates containing modified-brilliant green agar and xylose lysine tergitol 4 (XLT4) agar and incubated (37°C, 24 h). From each plate, three colonies with morphologic characteristics that suggested *Salmonella* genus (28) were inoculated in tubes containing triple-sugar-iron agar (TSI) and lysine-agar (LIA) (presumptive biochemistry tests) and incubated (37°C, 24 h).

Serotyping

After biochemical confirmation, slide agglutination tests were performed using somatic and flagellar polyvalent *Salmonella* antisera (poli-O, poli-H, and poli-D). Positive samples in slide agglutination tests were inoculated in tubes containing nutrient agar and sent to the laboratory of Enterobacteria of the Instituto Oswaldo Cruz – IOC/FIOCRUZ (Manguinhos, Rio de Janeiro, Brazil) for further serotyping.

Pulsed-Field Gel Electrophoresis (PFGE)

Positive samples in slide agglutination tests were also inoculated in tubes containing nutrient agar and sent to the "Laboratory of Veterinary Bacteriology, FCAV, UNESP, Jaboticabal Campus, Brazil," where they were subtyped by a standardized rapid Pulsed-field Gel Electrophoresis protocol used by laboratories in PulseNet, as described previously (29). Chromosomal DNA was digested with XbaI. Electrophoresis conditions consisted of a initial switch time of 2.2 s and a final switch time of 54.2 s (length of time the electrical field is applied on each direction) at a gradient of 6V cm^{-1} and an included angle of 120°. The gels were electrophoresed for 18 h.

Physical Examination and Laboratory Analysis

The health status of the lactating buffaloes was verified by physical examination, performed at the same time that rectal swab samples were collected. Their feces were analyzed for signs of diarrhea, blood, and mucus. Fecal consistency scores were determined as 0, normal (firm); 1, mild diarrhea (soft); and 2, and moderate to severe diarrhea (liquid) (30). The degree of dehydration was estimated as 0, absent (normal skin turgor and bright eyes); 1, mild (skin turgor slightly decreased and eyes not retracted); and 2, moderate to severe (skin turgor decreased and eyes retracted) (30). Rectal body temperature was measured. Also, the health status was verified by hemogram interpretation, being that blood samples were collected at the same time that rectal swab samples were performed. Blood sampling was performed by puncture of the jugular vein using a vacuum collection system (25 × 8 mm needles), after local antisepsis with iodized alcohol. Blood samples were collected into siliconized plastic tubes containing EDTA (BD Vacutainer, 4.0 ml). The health status of the pigs was verified by visual inspection and by feces inspection, performed at the same time that rectal swab samples were collected.

Hemogram, including red blood cell count (RBC), hemoglobin concentration (HGB), packed cell volume (PCV), and total white blood cell count (WBC) was performed using automated hematology pocH-100iV Diff analyzer (Sysmex Corporation, Kobe, Japan). Differential WBC count was performed on blood smear stained with modified Rosenfeld dye by optical microscopy (31). Normality was evaluated by comparing the results of hemogram to those described in literature for adult lactating buffaloes (32).

TABLE 1 | Identification of *Salmonella* serotypes isolated from feces of buffaloes and pigs, as well as the profiles of *S.* Agona, *S.* Senftenberg, and *S.* Schwarzengrund identified using PFGE analysis.

Serotypes	PFGE profile	Animal category	No of positive animals/no of tested animals (% of positive animals)	No of positive animals per serotype/no of tested animals (% of positive animals)
Salmonella Agona	1	Lactating buffalo	2/25 (8.0%)	2/25 (8.0%)
		Pregnant sow	1/32 (3.13%)	3/32 (9.39%)
		Gilt	1/32 (3.13%)	
	2	Weaned pig	1/32 (3.13%)	
Salmonella Senftenberg	3	Pregnant sow	2/32 (6.25%)	14/32 (43.75%)
		Lactating sow	2/32 (6.25%)	
		Gilt	2/32 (6.25%)	
		Boar	2/32 (6.25%)	
		Weaned pig	6/32 (18.75%)	
Salmonella Schwarzengrund	4	Lactating sow	2/32 (6.25%)	2/32 (6.25%)

RESULTS

Salmonella Serotypes Isolated

Bacteriological isolation showed that 2 (8.0%) of the 25 rectal samples collected from Jafarabadi lactating buffaloes were positive for *S.* Agona (**Table 1**).

In pigs, 19 (59.4%) of the 32 rectal samples collected were positive for *Salmonella*. From the positive samples, 3 (15.8%) were *S.* Agona, 14 (73.7%) were *S.* Senftenberg, and 2 (10.5%) were *S.* Schwarzengrund. Additionally, *Salmonella* was detected in more than one category of a pig inside the production cycle of the farm, being positive in 3/5 pregnant sows (60%), 4/5 lactating sows (80%), 3/8 gilts (37.5%), 2/3 boars (66.7%), and 7/11 weaned pigs (63.6%) (**Table 1**).

Pulsed-Field Gel Electrophoresis (PFGE) Analysis

PFGE analysis identified two profiles (profiles 1 and 2) of *S.* Agona, one profile (profile 3) of *S.* Senftenberg and one profile (profile 4) of *S.* Schwarzengrund. Profile 1 of *S.* Agona revealed a indistinguishable PFGE pattern between the isolates identified in two categories (pregnant sow and gilt) of pigs. This same profile was also identified in the two lactating buffaloes. Profile 2 of *S.* Agona was only identified in one animal, a weaned pig (**Table 1** and **Figure 1**).

The single profile identified for *S.* Senftenberg (profile 3) revealed a indistinguishable PFGE pattern between the isolates identified in all animal categories (sows, gilts, boars and weaned pigs) of pigs. The single profile identified for *S.* Schwarzengrund (profile 4) also revealed a indistinguishable PFGE pattern between the isolates identified in two lactating sows (**Table 1** and **Figure 1**).

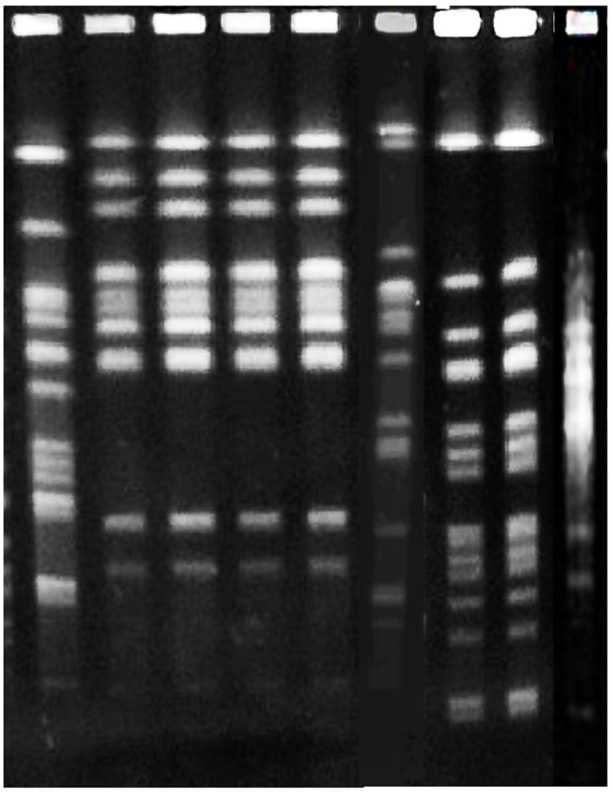

FIGURE 1 | PFGE profiles of *S.* Agona, *S.* Senftenberg and *S.* Schwarzengrund isolated from feces of buffaloes and pigs. PC: positive control (pulse marker, 50–1,000 kb, Sigma-Aldrich); 1: Profile 1—*S.* Agona isolated from a lactating buffalo; 2: Profile 1—*S.* Agona isolated from a lactating buffalo; 3: Profile 1—*S.* Agona isolated from a pregnant sow; 4: Profile 1—*S.* Agona isolated from a gilt; 5: Profile 2—*S.* Agona isolated from a weaned pig; 6: Profile 3—*S.* Senftenberg isolated from a weaned pig; 7: Profile 3—*S.* Senftenberg isolated from a boar; 8: Profile 4—*S.* Schwarzengrund isolated from a lactating sow.

Health Status

The health status of the lactating buffaloes was accessed by physical examination and hemogram analysis. In this sense, all buffaloes, both *Salmonella*-positive ($n = 2$) and *Salmonella*-negative ($n = 23$) animals, did not present clinical signs of salmonellosis, as well as did not present alterations in the hemogram (**Table 2**).

The health status of the pigs, both *Salmonella*-positive ($n = 19$) and *Salmonella*-negative ($n = 13$) animals, was verified by visual inspection and by feces inspection. In this sense, all animals were asymptomatic (no clinical signs) at the time of swab collection.

DISCUSSION

Cross-Species Transmission of *Salmonella* Agona Between Asymptomatic Buffaloes and Pigs

Molecular typing techniques, such us ERIC-PCR and PFGE, are an important method to distinguish different bacterial isolates and are useful to identify the origins of bacteria (33). In our study, *S.* Agona was isolated from two lactating buffaloes, a pregnant sow and a gilt, that generated an identical PFGE pulsetype (Profile 1), revealing a indistinguishable PFGE pattern between these isolates (**Table 1** and **Figure 1**). In this sense, these results bring evidence of a potential cross-species transmission of *S.* Agona between buffaloes and pigs, an important finding, considering that these species can act as asymptomatic reservoirs of *Salmonella* in dairy farms (1–3, 21, 22), contributing for the transmission of this disease to other species and also to humans (1, 3, 21, 22).

Concerning the possible forms of cross-species transmission of *S.* Agona, it is important to consider that buffaloes and pigs of all categories were released in the same paddock during the day, but did not have access to each other's feeders and water system. Therefore, although we have not isolate *S.* Agona from feces inside the paddock, positive isolation of this *Salmonella* serotype from rectal swabs of both species indicate that cross-species transmission likely occurred by fecal-oral route, probably linked to the ingestion of contaminated pasture due to animals shedding *Salmonella* through feces. Also, it was observed in our study that animals did not present clinical signs of Salmonelosis.

TABLE 2 | Physical examination and hemogram results of the two positive *S.* Agona lactating buffaloes ($n = 2$: LB1, LB2).

	Variables	Positive *S.* Agona LB		Neg.LB	Reference Values[a,b]
		LB1	LB2	Min–Max ($n = 23$)	
Physical examination	Rectal temperature	38.1	37.9	34.2–38.3	37.5–39.2
	Feces consistency score	0	0	0	0
	Degree of dehydration	0	0	0	0
Erythrogram	RBC ($\times 10^6/\mu$l)	6.47	6.81	5.49–8.14	4.70–10.60
	Packed cell volume (%)	29.4	34.9	27.9–38.5	26.1–45.3
	HGB (g/dl)	12.6	15.3	11.7–16.5	9.2–18.3
Leukogram	WBC (/μl)	9,400	14,500	7,600–11,700	5,000–14,700
	Lymphocytes (/μl)	3,196	4,470	2,464–6,120	2,400–9,100
	Segmented neutrophils(/μl)	4,794	8,689	2,754–6,555	1,800–9,100
	Band neutrophils (/μl)	0	0	0–105	0–500
	Monocytes (/μl)	0	149	0–380	0–1,200

RBC, red blood cell count; HGB, hemoglobin concentration; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC, total white blood cell count; [a](30): For feces consistency score and degree of dehydration; [b](32): For rectal temperature, erythrogram, and leukogram–reference values including minimum and maximum 90% CI.

Physical examination and hemogram results of Salmonella-negative lactating buffaloes (n = 23, Neg.LB), presented as the minimum (min) and maximum (max) values.

According to the literature (2), buffaloes infected with *Salmonella* serotypes may be asymptomatic, being that the major source of infection in the herd can be represented by asymptomatic older animals shedding bacteria through feces. In the other hand, a wide spectrum of *Salmonella* serotypes have been associated with a subclinical form of salmonellosis involving asymptomatic healthy pig carriers (21), and thereby these animals are a potential risk factor linked to cross-species transmission, since they can shed bacteria through feces (1, 22, 34).

Salmonella Infection in Asymptomatic Pigs Within the Pig Production Chain

Salmonella has been reported worldwide in pigs, with a great diversity of serotypes involved, and it is not uncommon to isolate more than one serotype on the same pig production cycle at a single farm (1, 8, 11–18). In our study, *S.* Agona, *S.* Senftenberg and *S.* Schwarzengrund were the serotypes detected from fecal samples of pigs (**Table 1**), that did not present diarrhea or other clinical signs of salmonellosis during visual inspection. These serotypes, being *S.* Agona (12, 13, 15, 16, 18), *S.* Senftenberg (18, 35), and *S.* Schwarzengrund (14, 15, 17), have already been isolated in pigs.

S. Agona isolates identified from the pregnant sow and the gilt generated an identical PFGE pulsetype (Profile 1), revealing a indistinguishable PFGE pattern between these isolates (**Table 1** and **Figure 1**), while *S.* Agona isolated from the weaned pig generated a different PFGE pulsetype (Profile 2) (**Table 1** and **Figure 1**). Since we sampled each animal at only one timepoint, finding only three animal with a positive result for *S.* Agona could have occurred due to the fact that intermittent shedding of *Salmonella* by pigs is common, and according to literature (36), this can interfere with monitoring and research programs on *Salmonella* infection and the determination of health status in animals. Otherwise, the presence of latent undetectable carriers among infected pigs is a common characteristic in the epidemiology of *Salmonella* (37), and is another fact that must be considered.

S. Senftenberg, isolated from four sows, two gilts, two boars, and six weaned pigs, generated an identical PFGE pulsetype (Profile 3), revealing a indistinguishable PFGE pattern between these isolates (**Table 1** and **Figure 1**). In this sense, pig colonization, in our study, could have occurred by horizontal and vertical transmission, or yet by a combination of vertical and horizontal transmission, which is a permanent cycle of contamination on farms (22, 24). It is known that transmission of *Salmonella* between pigs occurs mainly via the fecal-oral route (38) and therefore horizontal transmission between pigs could be occurring, since all animals shared the same installations during the day (paddocks) and during the night (all categories were confined together). Also, it is unlikely that the buffaloes are somehow participating in the transmission of this serotype, since it has not been detected in this specie. In this sense, it is important to consider that, according to literature, serovar Senftenberg has been isolated in pig feed (39), indicating that the consumption of contaminated feed could be the infection source for pigs but not for buffaloes. This can be reinforced by the fact that buffaloes did not have access to pigs feeders and water system.

S. Schwarzengrund, isolated from two lactating sows, generated an identical PFGE pulsetype (Profile 4), also revealing a indistinguishable PFGE pattern between these isolates (**Table 1** and **Figure 1**). The fact that *S.* Schwarzengrund was not identified within all the production chain inside the farm, does not mean that this *Salmonella* serotype is restricted to lactating sows. For instance, a study (22) reported that the sensitivity of fecal samples collected on-farm was particularly poor (prevalence of 0%) when comparing to lymph node samples (prevalence of 12.2%) of the pigs at the abattoir. Furthermore, the isolation of *S.* Schwarzengrund in pigs, added to the other two serotypes isolated in pigs in our study, shows the importance of *Salmonella*

in this specie and reinforces the theory that a great diversity of serotypes can be involved in the infection cycle inside a single farm.

The results of this work confirmed a potential transmission of *S.* Agona between buffaloes and pigs. Also, evidenced a potential *Salmonella* infection within the pig production phases. Therefore, considering the epidemiological importance of buffaloes and pigs in the cycle of *Salmonella* infection, the results presented reinforce the importance of the correct establishment of preventive health strategies in farms, in special the importance of avoiding contact between buffaloes and pigs when raised in the same farm, since cross-species transmission can occur, increasing the risk of spreading the disease.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by The Ethics Committee on Animal Use of Faculdade de Ciências Agrárias e Veterinárias, UNESP (Protocol no 010885-08). Written informed consent for participation was not obtained from the owners because at the time of the execution of the project, the written consent was not a requirement of the Ethics Committee on Animal Use of "Faculdade de Ciências Agrárias e Veterinárias, UNESP". Therefore, only a verbal consent was given.

AUTHOR CONTRIBUTIONS

AS was responsible for the conception of the study, experimental design, collection of samples in the farm, part of laboratory work, data analysis and interpretation, and manuscript writing. DS and JF were responsible for the conception of the study, experimental design, part of laboratory work, data analysis and interpretation, and manuscript revision. RM was responsible for the conception of the study and part of the laboratory work. LP and KS were responsible for the collection of samples on the farm. CS and DR were responsible for part of the laboratory work. SR was responsible for data analysis and interpretation and manuscript revision. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the São Paulo Research Foundation (FAPESP) (Grant process numbers: #2008/50388-7 and #2009/12350-0).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 24 November 2020
doi: 10.3389/fvets.2020.542338

Selectivity of Leguminous Trees by Water Buffaloes in Semi-intensive Systems

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Water buffaloes (*Bubalus bubalis*) manifest different levels of selectivity for different pastures and forages. Knowledge of feed selectivity is important to facilitate the design of efficient production systems that take into account optimal animal welfare. In this study, the selectivity of nine 18-month old female water buffaloes for *Leucaena leucocephala*, *Albizia lebbeck*, *Gliricidia sepium*, and *Moringa oleifera* was evaluated. After 12 h of grazing *Megathyrsus maximum*, the animals were housed in individual shelters and 1.2 kg of leaves from each of the four tree species were offered to the animals simultaneously. The selectivity, measured as the intake of dry matter (DM), was highest for *A. lebbeck* (mean = 0.34 kgDM, SD = 0.05 kg), followed by *L. leucocephala* (mean = 0.30 kgDM, SD = 0.03 kg), *M. oleifera* (mean = 0.11 kgDM, SD = 0.05 kg), and *G. sepium* (mean = 0.10 kgDM, SD = 0.02 kg) ($P < 0.01$). The crude protein intake was highest for *A. lebbeck and L. leucocephala*. Notably, the less selected leaves were those of *G. sepium* and *M. oleifera*. This study suggests that the inclusion of *A. lebbeck* and *L. leucocephala* in silvopastoral systems may increase both the consumption and well-being of water buffaloes in the tropics.

Keywords: selectivity, feeding behavior, buffaloes (*Bubalus bubalis*), consumption, trees leaves, semi-intensive system

OPEN ACCESS

Edited by:
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Reviewed by:
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†Deceased, 6 August 2016. Contribution: Research Design

Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *12 March 2020*
Accepted: *16 September 2020*
Published: *24 November 2020*

Citation:
Galloso-Hernández MA, Rodríguez-Estévez V, Alvarez-Díaz CA, Soca-Perez M, Dublin DR, Iglesias-Gómez J and Guelmes LS (2020) Selectivity of Leguminous Trees by Water Buffaloes in Semi-intensive Systems. Front. Vet. Sci. 7:542338. doi: 10.3389/fvets.2020.542338

INTRODUCTION

The importance of buffaloes (*Bubalus bubalis*) as a productive species has increased worldwide by 2% in recent years with a total population of 202 million (1). For example, Cuba has a population of 6×10^4 buffaloes (2, 3) with an annual increase of 13.7% and comparatively less than Brazil with 3×10^6 buffaloes (4). In Argentina, the population amounts to 8.5×10^4 with an annual growth of 13.1% (5).

The advantages of using buffaloes in tropical environments are the resistance to heat stress under shade (6, 7), and the fact that it allows this species to maintain birth rates above 80%, which is higher than cattle in similar conditions (8). However, the milk production per hectare (ha) is lower than cattle due to the low stocking rates (0.6–0.8 animals/ha) currently used to breed buffaloes (8, 9). The capacity of buffaloes to digest highly fibrous diets (10, 11) makes the use of trees an attractive strategy for the incorporation of proteins in the diet of these animals (12–15).

Tropical tree species are included in pastures in the form of silvopastoral systems. The incorporation of *Leucaena leucocephala* (13), *Albizia lebbeck* (16), *Gliricidia sepium*, and *Moringa oleifera* (17, 18) in silvopastoral systems improves the quality of feed for ruminants and decreases the impact of environmental stressors on the animals (6). The use of trees as a source of feed for ruminants has been ignored due to the limited understanding of their positive impact on production systems (19). Leguminous trees are a source of feed in sustainable livestock farming in the dry season (19–22). A good selection of trees maximizes the benefits of agroforestry systems (13, 23, 24). Leguminous trees (*L. leucocephala, A. lebbeck, G. sepium, and M. oleifera*) have perennial leaves and fruits with high nutritional value. Ruminal evaluations showed that diets comprised of pasture and tree leaves have a higher rate of degradation (i.e., organic material, dry matter, crude protein, gross energy). Leucaena trees, for example, have the highest rumen by-pass protein supply. This digestibility of the protein is 63% in *L. leucocephala* and around 60% in other tree species (25–28), which decreases the amount of commercial concentrates being used (21, 29, 30). According to Ku Vera et al. (26), we need to deepen our understanding through the manipulation of different diets in ruminants including the use of leguminous trees and grasses in tropical conditions. The branches of leguminous trees also contain condensed tannins with antiparasitic properties (31) that could reduce enteric CH4 emissions (32).

The productivity of buffaloes has been evaluated in silvopastoral systems in tropical countries including Cuba (8, 22, 33), and Brazil (34). Silvopastoral systems, combined with rusticity and high productive indexes (13, 21), improve the productive performance of buffaloes per ha by increasing the daily weight gain and milk production when compared to conventional systems (35). Previous studies have tested the effect of individual leguminous trees on ruminant metabolism (12, 25, 36). However, in natural conditions, animals select a mixture of foliage from different trees. The aim of this study was to evaluate the selectivity of water buffaloes for four tree species (*L. leucocephala, A. lebbeck, G. sepium, M. oleifera*) that are commonly used in silvopastoral systems as demonstrated in the aforementioned studies.

MATERIALS AND METHODS

Ethics Statement

The experiment received the approval of the Scientific Council and the Ethics Committee of the "Indio Hatuey" Grass and Forage Experimental Station, Matanzas, Cuba. This was an observational study that did not involve any harm or cruelty to animals.

Animals and Study Site

Clinically healthy 18-month old female water buffaloes ($n = 9$), with an average body weight of 373.77 kg, and daily weight gain of 362 g were used in the study. Before being included in the experiment, the animals were treated with ivermectin (1 μg/kg, Labiomec, Labiofam, Habana, Cuba) to ensure that their consumption behavior is not affected by intestinal parasites.

The study was conducted between August and September of 2008 in the municipality of Périco, Matanzas, Cuba, located at 22°48′7″ of latitude north and 81°1′ of longitude west and 19.01 m above sea level. The experimental phase was carried out on hydrated red ferralitic soil with medium fertility, moderately acidic (pH 5.60), low in phosphorus content (2.43 mg/100 g), and containing 0.18% total nitrogen, 3.2 % organic matter, calcium predominates (11.84 mEq/100 g) among the exchangeable cations and the cation exchange capacity was slightly low (19.21 mEq/100 g) as previously described (37). The climate of the region is tropical, seasonally humid, with an annual average temperature between 24.3°C and 33.4°C, and relative humidity of 80%. The annual rainfall is 1,331 mm, where 79.8% of it occurs between May and October (38).

Twelve paddocks, each measuring 0.54 ha, were used through a rotation of 3 days of occupation and 42 days of rest. In the morning, the animals were moved to the paddocks, where the availability of feed was 2.45 Ton/ha of dry matter (DM), and from 18:00 h the animals had access to the leaves of the four species in the feeders (**Figure 1**). Grass availability was determined by the visual estimation method and the botanical composition was measured as previously described (6, 39). During the experiment, we collected five grass samples and one sample per tree leaf type. The feeding regime included the grazing of *Megathyrsus maximum* in paddocks and nocturnal supplementation with *Saccharum officinarum* (sugar cane) (0.52 kg DM/animal) and the PCN002 commercial feed for growing animals (MINAG, Habana, Cuba) (**Table 1**) (0.44 Kg DM/animal). In the grazing area (**Figure 1A**), free access to water for wallowing and natural shade by *Dichrostachys cinerea* (Marabú) was available in the wallowing areas. Drinking water and mineral salts were provided *ad libitum*.

Experimental Design

In this study, the preference and consumption of fresh tree leaves were determined in a group of water buffaloes. *Leucaena leucocephala*, *Albizia lebbeck*, *Gliricidia sepium*, and *Moringa oleifera* were offered simultaneously (1.2 kg/animal of each fresh leaf type) to the animals. The buffaloes had no previous consumption experiences of the leguminous tree species studied. The trees were pruned and only the leaves that sprouted after 60 days were used in the experiment. Leaves of the trees were cut at a height of 1.7 meters, and the pods and stems were excluded. Fresh leaves were collected daily between the hours of 9:00–11:00. The trees were not irrigated nor treated with agrochemicals.

The animals were fed for 15 days with tree leaves and a base diet (**Table 1**). Experimental measurements were made daily from day 16 to day 32. After returning from the paddock, the animals were separated into individual shelters and each type of leaf was randomly placed separately in one of four feeders (each feeder measured 50 × 90 × 30 cm).

We used the repeated measured model shown in equation (1) below:

$$\mathbf{Y_{ijklm} = m + T_i + P_j + A_j + e_{ijklm}}$$

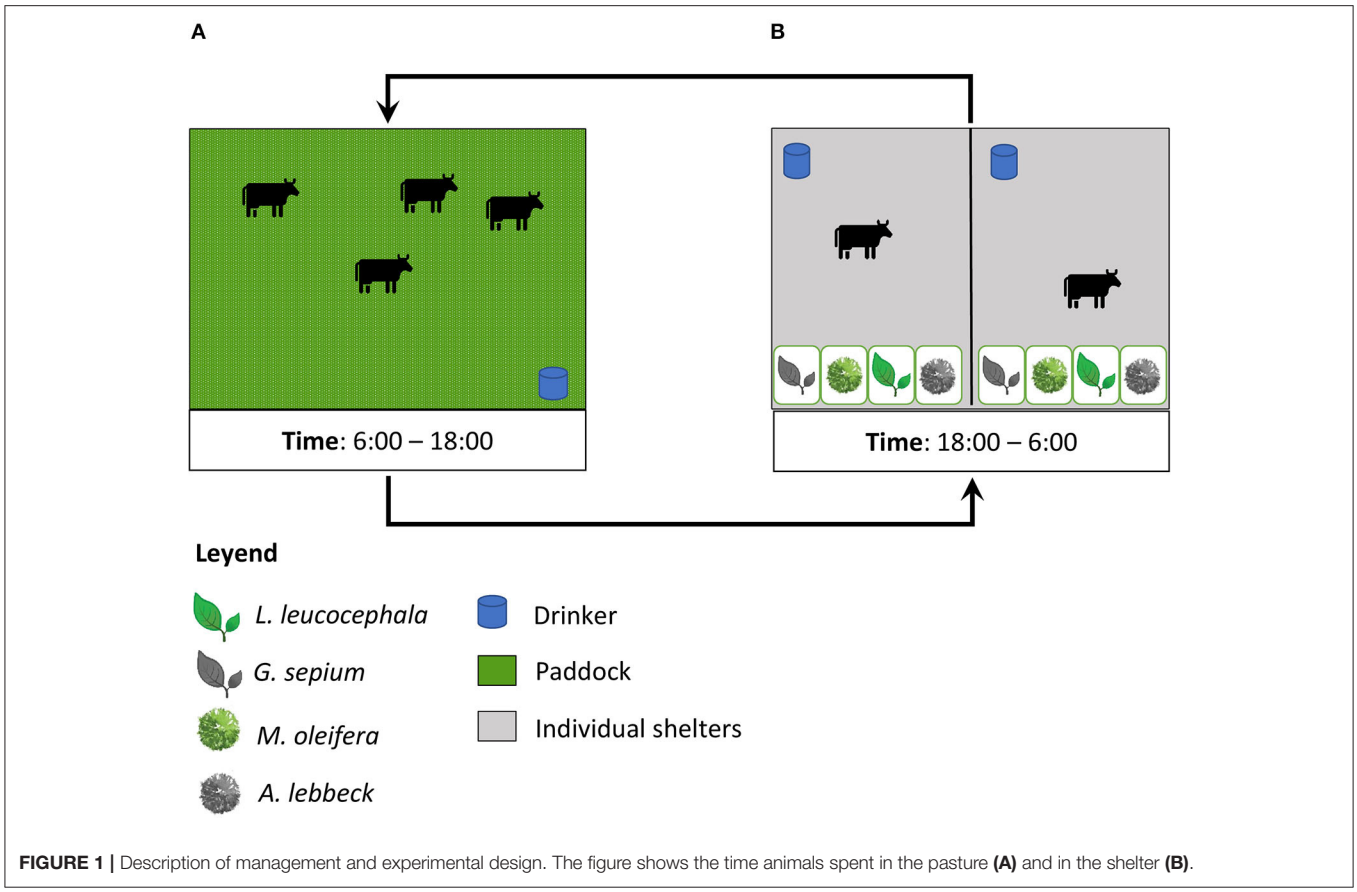

FIGURE 1 | Description of management and experimental design. The figure shows the time animals spent in the pasture **(A)** and in the shelter **(B)**.

TABLE 1 | Chemical composition of ingredients in the diet.

	DM* %	CP (g/kg)	CF (g/kg)	GE Mcal/kg DM	Ca (g/kg)	P (g/kg)
Chopped Sugar cane (3 mm) *S. officinarun*	26	28	240	2.19	0.6	0.1
Comercial feed	88	110	430	2.55	1.7	0.2
M. maximus	23	80	180	1.81	1.2	0.1
L. leucocephala	26.02	205	282	2.25	2.3	2.5
A. lebbeck	32.68	236	327.6	2.41	0.88	1.49
G. sepium	28.79	246	283	2.53	1.7	2.1
M. Oleifera	28.7	265.6	324.8	1.63	0.44	2.59

**Dry matter (DM %), crude protein (CP, g/ KgDM), crude fiber (CF, g/ kgDM), Gross Energy (gross energy, Mcal/kgDM), calcium (Ca, g/kg DM), and phosphorous (P, g/kg DM).*

where:

Y_{ijklm} = represented the m-n measurement made in the l-n square the j-n period in the i-n treatment in the k-n sampling within the j-n period.

m = population means or general intercept.

T_i = fixed effect of the i-n treatment (i = 1: Ll, 2: Al, 3: Gs, 4: Mo).

P_j = fixed effect of the i-n day (i = 1,….. 16).

A_i = fixed effect of the i-n animal (i = 1, 2, 3....9)

e_{ijklm} = random residual associated with the m- n measurement. ∼ N (0, s2).

Analysis of Consumption and Chemical Composition of Fodder

The consumption of each leaf type was measured by subtracting the amount left in the feeders from the initial amount of leaves placed. For the weighing of fresh leaves, a digital scale with a sensitivity of 0.01 kg was used. Laboratory analysis was

carried out at the beginning of the experiment to determine the DM, crude protein (CP), crude fiber (CF), and gross energy (GE). All procedures of feed analysis were performed as described in the manual "Official methods of analysis of AOAC international" (40).

The DM content of the leaves was determined individually in the laboratory of the Indio Hatuey Grass and Forage Experimental Station as described previously (41). To estimate the DM content, 500 g of homogenized grass samples were dried in a forced-air oven at 60°C for 48 h. The weight of each leaf sample was determined before and after the dehydration process (40) using an analytical balance of sensitivity 0.001 kg. After dehydration, the leaves were ground to a size of 2 mm and stored in amber glass jars.

The CP was calculated by the Kjeldahl method (42). CF was estimated by the Van Soest method (43, 44) briefly described by Garcia et al. (17). The GE was calculated with calorimetric methods using benzoic acid as the internal standard (45). This analytical procedure was performed with an adiabatic calorimetric pump (Model 1341EB, Parr instrument, Illinois, United States).

Statistical Analysis

The results were analyzed using the SPSS program version 22 (IBM Corp, New York, United States). An analysis of variance (ANOVA) was applied to determine whether there were any statistically significant differences between the measurements. Each buffalo was considered as an experimental unit (9) during the experimental period of 16 days. The weight of the feed (leaves and other supplements) before and after its placement in the feeders was evaluated individually. A Pearson correlation test was performed for the consumption of dry matter for the four leaf types.

RESULTS

Water Buffaloes Leaf Type Preferences

There was a daily variation in the consumption of the four types of leaves studied (**Figure 2A**). The leaves of *A. lebbeck* (0.34 kgDM) and *L. leucocephala* (0.30 kgDM) were the most consumed, respectively, while the leaves of *M. oleifera* (0.11 kgDM) *and G. sepium* (0.10 kgDM) were the least consumed (**Figures 2A**, **3A**). Each animal consumed 0.85 kg of DM of leaves daily ($P < 0.01$). The consumption of *A lebbeck* and *L. leucocephala* was always the most consumed, but it was observed that this amount reduced when the buffaloes consumed higher quantities of *G. sepium* and *M. oleifera*.

Contribution of Legumes to the Buffalo Diet

The analysis of the chemical composition revealed differences between the evaluated species (**Table 1**). The DM content was

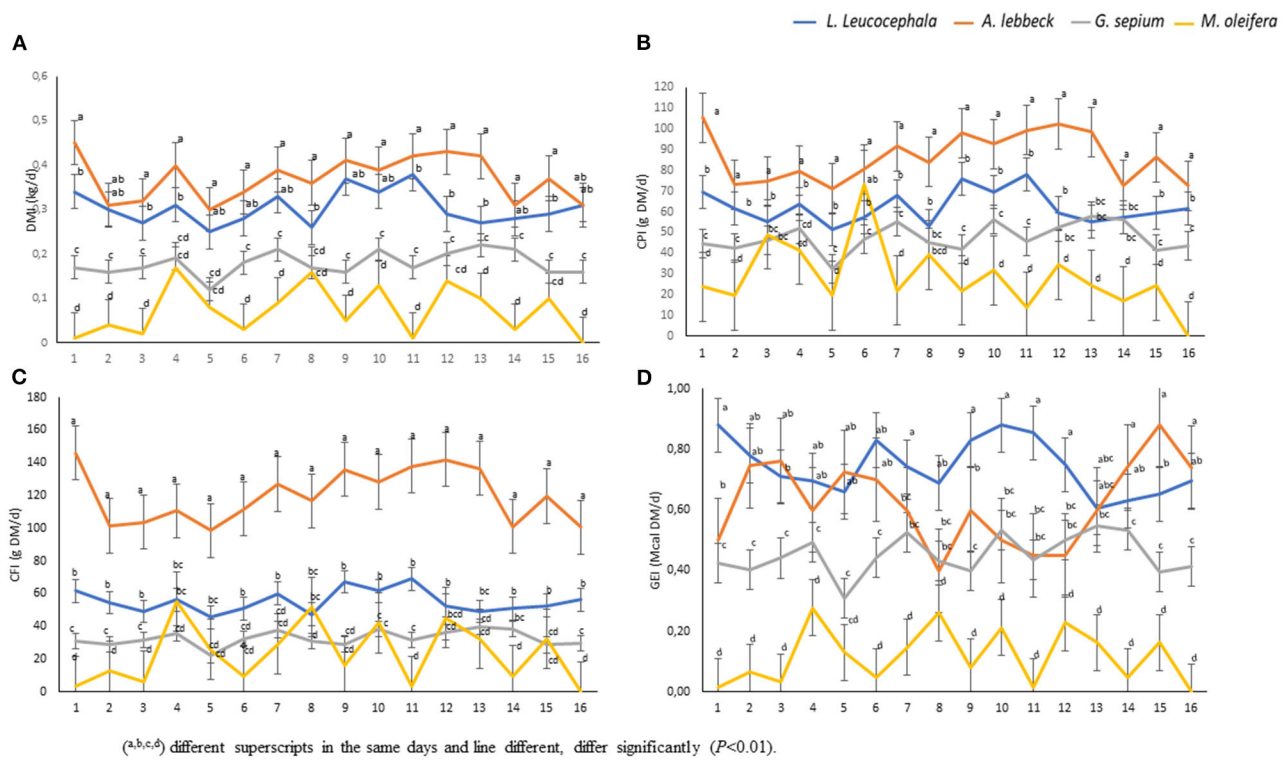

FIGURE 2 | Consumption and overall nutritional contribution of the leaves. The statistical differences in the consumption of dry matter of the leaves are shown, as dry matter intake (DMI) **(A)**; crude protein intake (CPI) **(B)**; crude fiber intake (CFI) **(C)**; and gross energy intake (GEI) **(D)**. (Duncan, $P < 0.01$). $N = 9$ during 16 days of evaluation ($P < 0.01$).

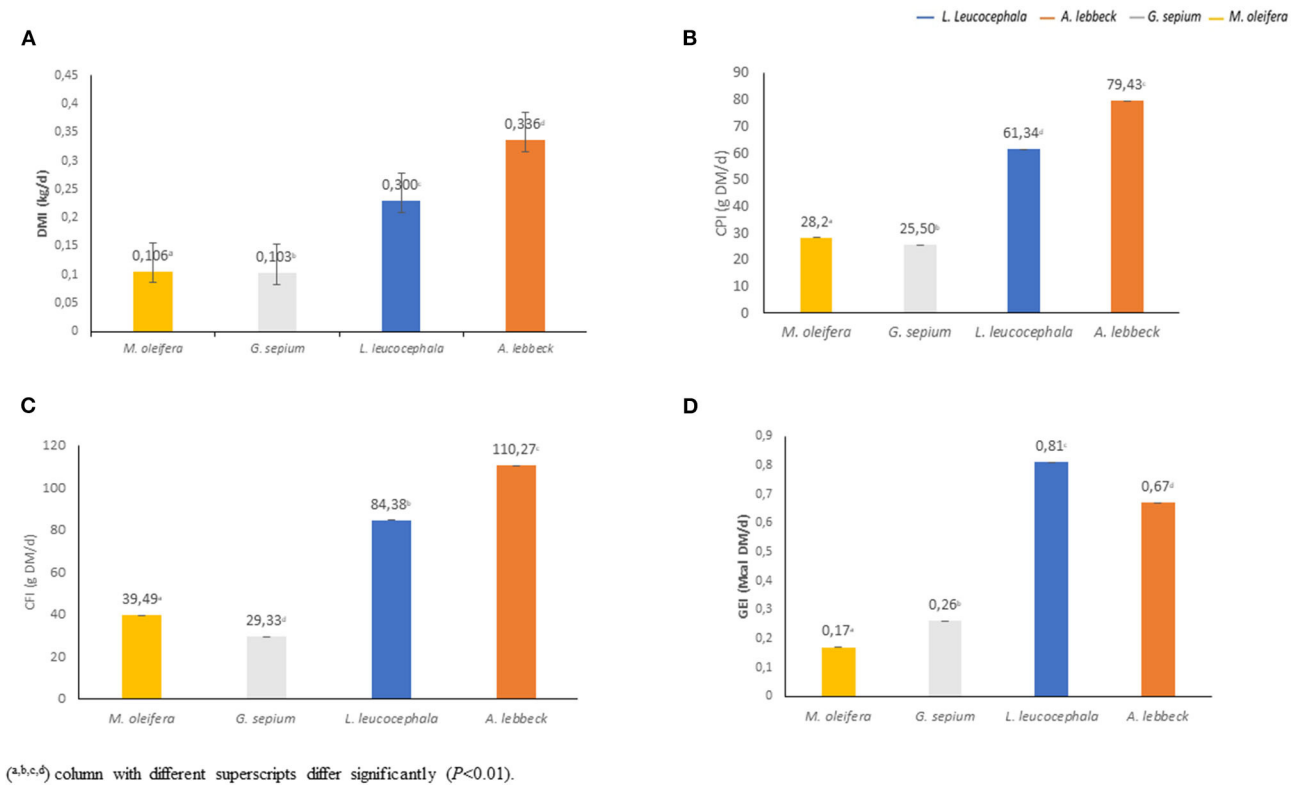

FIGURE 3 | Nutritional contribution of each leaf type. The figure represents the average contribution (per animal/day) of dry matter intake (DMI) **(A)**, crude protein intake (CPI) **(B)**, crude fiber intake (CFI) **(C)** and gross energy intake (GEI) **(D)** ($P < 0.01$).

lower in *M. oleifera* and *G. sepium*. Notably, *M. oleifera* had the lowest GE values. *L. leucocephala* and *M. oleifera* were the species with the lowest and highest CP, respectively. CF content was lower in *G. sepium* and *L. leucocephala* but higher in *M. oleífera* and *A. lebbeck* (**Table 1**). The contribution of each of the species in terms of feed intake was different (**Table 2** and **Figures 2A–D**). Of the leaves consumed, DM content differed between the four tree species during the period evaluated (**Figure 2A**). The CPI was highest in *A. lebbeck* and *L. leucocephala* followed by *M. oleifera* and *G. sepium* ($P < 0.01$; **Figures 2B**, **3B**). The CFI was different between treatments. *A. lebbeck* had the highest CF content followed by *L. leucocephala*, *M. oleifera*, and *G. sepium*, respectively ($P < 0.01$; **Figures 2C**, **3C**). However, the consumption of GE was highest in *A. lebbeck* followed by *L. leucocephala*, *G. sepium*, and *M. oleifera,* respectively ($P < 0.01$; **Figures 2D**, **3D**). No differences were found in the consumption of calcium, phosphorous, and organic material present in the four types of leaves.

The average intake of fresh leaves was 2.91 kg/day and represents a daily DM consumption of 0.85 kg in this study (**Table 2**). The CPI of leaves was 194.48 g /animal/d; CFI was 258.48 g/ animal/d and GEI was 1.92 Mcal/animal/d.

The total DM Intake (leaves + *M. maximum* + *S. officinarum* + commercial feed) in this study was 10.62 KgDM/animal corresponding to 2.83 % body weight, and 936.45 g of CPI/animal, CFI = 2,160.99 g/animal; GEI 20.15 Mcal /animal, Ca = 12,86 g/animal and phosphorous = 2,76 g/animal (**Table 2**).

The DMI between *L. leucocephala* and *A. lebbeck* showed significant correlation (Pearson $r = 0.54$, $P < 0.01$); DM consumption of *G. sepium* and *M. oleifera* showed a positive correlation (Pearson $r = 0.26$; $P < 0.01$). Notably, the correlation of DMI between *L. leucocephala* and *M. oleifera* was negative (Pearson $r = -0.28$; $P < 0.01$); *A. lebbeck* and *G. sepium* had a value of $r = 0.32$. DMI of the most preferred and least preferred legume leaves (*L. leucocephala* + *A. lebbeck* vs. *G. sepium* + *M. oleifera*) showed a negative correlation (Pearson $r = -0.43$; $P < 0.01$).

DISCUSSION

Consumption of the leguminous trees was higher in *L. leucocephala* but provided less DM, CP and CF when compared to *A. lebbeck*. In this study, *A. lebbeck* was the leaf most consumed on the basis of DM, except for GE, where *L. leucocephala* was the most consumed species in the diet.

Previously, an evaluation in the paddock was conducted on bovine browsing behavior in an arboretum of 60 tree species. The findings showed a preference for *L. leucocephala* and *A. lebbeck* in cattle (46). This evaluation was repeated with buffaloes resulting in a similar preference for *L. leucocephala* (24). According to

TABLE 2 | Principal nutrient composition in the diet.

	Nutrient intake of the diet*							
	Quantity(kg)	**DMI (kg/d)**	**CPI (g DM/d)**	**CFI (g DM/d)**	**GEI (Mcal DM/d)**	**GEI (Mcal DM/kg)**	**Cal(g DM/d)**	**PI(g DM/d)**
Chopped Sugar cane (3mm) *S. officinarun*	2.00	0.52	14.56	124.80	1.14	2.19	0.31	0.05
Comercial feed	0.50	0.44	48.40	189.20	1.12	2.54	0.75	0.09
M. Maximus	38.37	8.83	706.01	1,588.52	15.97	1.80	10.59	0.88
Subtotal		9.79	768.97	1,902.52	18.23		11.65	1.02
%		92.05	79.81	88.04	90.47		90.61	36.98
L. Leucocephala	1.15	0.30	61.34	84.38	0.67	2.23	0.69	0.75
A. lebbeck	1.03	0.34	79.44	110.27	0.81	2.38	0.30	0.50
G. Sepium	0.36	0.10	25.50	29.33	0.26	2.60	0.18	0.22
M. Oleifera	0.37	0.11	28.20	34.49	0.17	1.54	0.05	0.28
Subtotal		0.85	194.48	258.48	1.92		1.21	1.74
%		7.95	20.19	11.96	9.53		9.39	63.02
Total	43.78	10.63	963.45	2,160.99	20.15		12.86	2.76

**Quantity (kg), dry matter intake (DMI, Kg/d), crude protein intake (CPI, g DM/d), crude fiber intake (CFI, g DM/d), gross energy intake (GEI, Mcal DM/d), gross energy intake per kg(GEI, Mcal DM/kg) calcium intake (Cal, g DM/d), and phosphorous intake (PI, g DM/d).*

García et al. (18), *M. oleifera* was a moderately-consumed plant. *A. lebbeck and L leucocephala* was the most consumed species, probably due to the ease of collecting these branches in the feeders. *L. leucocephala* has very small leaves which are easily licked from the bottom of the feeders while *A. lebbeck* and *G. sepium* are more resistant to this type of action (47). However, it was observed that the rate of wilting of *M. oleifera*, prevents its consumption by the animals. We could attribute the preference for *L. leucocephala* and *A. lebbeck* to the "feeding memory" (i.e., the tendency of animals to remember the information of feed for up to 3 years) effect, reported in sheep (48) and buffaloes (49). The difference in the consumption and acceptability of the tree leaves could be suggestive of the satisfaction of the needs of the appetite and not that of "hunger" (50, 51). Additionally, ruminants prefer leaf types as a supplement that rapidly provides the highest satiety level of nutrients (52, 53).

The DM content of the feed in ruminants is partially responsible for limiting its consumption and ingestion through short-term regulation along with the fast fermentation of the carbohydrate contents (10, 50). These results are indicative of the satisfaction of the animal's needs and coincide with what was reported by Mendez and Lima (54) who indicated that the voluntary consumption (expressed in the percentage of body weight) is 2.59–3.09%) in buffalos. Similar results were previously published by Paul and Lai (55).

Tree leaves (*L. leucocephala, A. lebbeck, G. sepium*, and *M. oleifera*) contributed 194.48 g DM to the daily CPI. This represents 20.19% of the protein even though the trees only provided 7.95% of the DM in the diet. The rest of the diet provided 768.97 g CPI/animal/day (79.81% of diet). Paul and Lai (55) reported a daily CPI of 298–310 g in the diet of female buffaloes with a similar body weight of 300–350 kg and daily weight gain of 250 g/day, while being fed with different proportions of feed in India. The value of the proportion of CPI due to the ingestion of tree leaves in this study was similar to a study in bovines in Cuba (21, 45, 56). Of the four types of leaves, *A. lebbeck* was the one that contributed the most to the protein needs of buffaloes and was sufficient to cover all the requirements of the animals used. Valenciaga et al. (25, 57) estimated in Cuba that a DM intake of 9.95 and 10.25 kg DM in buffaloes with 600 kg of BW was needed.

Delgado et al. (12) reported an apparent digestibility above 60% for trees such as *G. sepium* and *L. leucocephala* in similar conditions in Cuba. In this sense, Albores-Moreno et al. (58) reported high voluntary consumption in cattle (10.26–12.48 kg of D/animal/day) of legumes and found that the secondary metabolite content of plants did not interfere with the voluntary intake. Additionally, Barros-Rodríguez et al. (28) report that up to 50% of *L. leucocephala* can be included in the diet. These criteria could explain the favorable consumption in favor of *L. leucocephala* and *A. lebbeck*, in the same way, that it can be attributed to the digestibility as reported by Delgado et al. (12). However, they do not explain why *G. sepium* and *M. olifera* were scarcely consumed.

Notably, the lower intake of *M. oleifera* could be related to the effects of this plant on the ruminal flora, volatile fatty acids, and ruminal ammonium concentration (59). This legume is low in fiber with higher degradability, gas production, short chain fatty acids production, and lower methane emissions as compared to wheat straw.

An *in vitro* study with an *M. oleifera* leaf extract as an alternative to monensin in sheep diets, found similar effects on the ruminal parameters (ruminal degradability, ammonia concentrations, and gas production) (60). The lower consumption of *M. oleifera* could also depend on the concentration of anti-nutritional factors (condensed tannin,

tannins which are precipitants of proteins, terpenoids, and total sterols) (18, 31) and the ability of this leaf type to wilt, rather than its nutritional value. Previous studies attributed the texture, anti-nutritional factors and the apparent digestibility (17, 18, 32, 48) to the speed with which the branches lose qualities after being cut (61). It could also be attributed to the vegetative state or age (60- days-old) in which the leaves of the plants were collected (62, 63), climatic conditions (13, 56), the animal category (48), the amount and nutritional composition of the tree resource (64), DM content and apparent digestibility (32, 65–67). However, there does not seem to be a direct relationship between the nutritional composition and the palatability of the leaves of leguminous trees, and the leaves of *L. leucocephala* and *A. lebbeck* may generate similar stimuli in animals, meaning they are consumed more.

Pearson's correlation was significantly negative between the two most consumed species (*L. leucocephala* and *A. lebbeck*) and the two least consumed (*G. sepium* and *M. oleífera*). In this sense, there was no correspondence between what was reported by Santana-Perez et al. (47) regarding the low correlations of consumption of *A. lebbeck* (stems with irregular barks) compared to *L. leucocephala*, *G. sepium*, and *M. oleifera* (smooth stems), attributing it to the physical characteristics of the stems.

Analysis showed a negative correlation between *L. leucocephala* and *M. oleifera* in the DM intake, similar results were obtained previously (17, 18) where during grazing they observed *M. olifera* being rejected in comparison with *L. leucocephala* and other leguminous trees. Santana-Perez et al. (47) found differences in voluntary consumption in sheep and cattle, attributing it to the differences between the maximum diameters of the stems consumed. With *G. sepium*, *L. leucocephala*, *M. oleifera*, and *A. lebbeck* being the most consumed, respectively, the sheep did not consume the stems of *G. sepium*, attributing this phenomenon to the aversive stimulus generated by this plant.

CONCLUSIONS

There were significant differences in the consumption and acceptability of the leaves of the tree species. The highest consumption of leaves was that of *L. leucocephala* and *A. lebbeck*, while *M. oleifera* and *G. sepium* species were consumed in lower quantities. The leaves of *A. lebbeck* contributed more nutritionally to the animals, although the amounts consumed were equal to *L. leucocephala*. This study helps to define which tree species could be used in production systems with buffaloes in the tropics by providing insights into buffaloe preferences for different leaves. We suggest that future studies explore the effects of secondary metabolites on feed intake.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the ethics committee of the Experimental Station: Indio Hatuey, Cuba.

AUTHOR CONTRIBUTIONS

All authors also participated equally in the design, preparation, and review of this research.

FUNDING

BIOMAS-CUBA PROJECT. COSUDE. For grant and support of the execution of these experiments and Experimental Station: "Indio Hatuey" (Financial support of this research in to the topic's of sustainable Livestock production).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 25 November 2020
doi: 10.3389/fvets.2020.522551

Thermoregulatory and Behavioral Responses of Buffaloes With and Without Direct Sun Exposure During Abnormal Environmental Condition in Marajó Island, Pará, Brazil

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OPEN ACCESS

Edited by:
Antonio Minervino, Federal University of Western Pará, Brazil

Reviewed by:
Alessandra Pelagalli, University of Naples Federico II, Italy
Antonio Borghese, International Buffalo Federation (IBF), Italy
Rejane Santos Sousa, Federal University of South and Southeast of Pará, Brazil

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Specialty section:
This article was submitted to Animal Nutrition and Metabolism, a section of the journal Frontiers in Veterinary Science

Received: *23 December 2019*
Accepted: *02 October 2020*
Published: *25 November 2020*

Citation:
Athaíde LG, Joset WCL, de Almeida JCF, Pantoja MHdA, Noronha RdPP, Bezerra AS, Barbosa AVC, Martorano LG, da Silva JAR and Lourenço Júnior JdB (2020) Thermoregulatory and Behavioral Responses of Buffaloes With and Without Direct Sun Exposure During Abnormal Environmental Condition in Marajó Island, Pará, Brazil. Front. Vet. Sci. 7:522551. doi: 10.3389/fvets.2020.522551

This study aimed to assess the effect of thermal-hydraulic variables in female buffaloes with or without direct solar exposure in a year of strong El Niño through behavior responses and infrared thermography to reinforce the environmental comfort indicators, in Marajó Island, Pará, Brazil. The experiment was carried out in Cachoeira do Arari municipality and 20 female Murrah buffaloes were randomly assigned to two groups: Group WS ($n = 10$) was kept in pickets with native trees. Group NS ($n = 10$) was kept in crush squeeze with no shade. Data on air temperature (AT, °C), relative air humidity (RH, %), wind velocity (WV, m/s), rectal temperature (RT), respiratory rate (RR), and body surface temperature (BST) were collected. Practical Buffalo Comfort Climatic Condition Index (BCCCI), practical Buffalo Environmental Comfort Index (BECI), Temperature and Humidity Index (THI) and Benezra's Thermal Comfort Index (BTCI) were obtained. Infrared thermography analysis was carried out with a FLIR T-series T640bx camera. Data on time spent grazing, ruminating, idleness, and in other activities were recorded. A significant difference in AT of ∼1°C was found between the groups at 6 a.m., 10 a.m. and 6 p.m. THI indicated emergency conditions. Female buffaloes were at danger PBCCCI conditions at 2 p.m. There was also significant difference for RT between treatments at 10 a.m., 2 p.m. and 6 p.m., whose values were higher ($P < 0.05$) for animals from NS Group, with the highest mean time at 2 p.m. Pearson correlation was significant and positive ($P < 0.01$) between RT mean and VUL, TI and ORB mean, maximum and minimum temperatures. The total time given to grazing was 518.2 min for the group NS and 629.5 min for the group WS. Rumination was more pronounced in the afternoon shift for the group NS. Buffaloes kept in a system with trees graze, ruminate and perform other activities with more intensity than animals raised in systems without access to shade, and tend to hyperthermia, mainly at 10 a.m. and 2 p.m., in Marajó Island, Pará, Brazil.

Keywords: amazon, ambience, ethology, physiology, thermography

INTRODUCTION

The Marajó Archipelago, Pará, Brazil, is the largest marine fluvial complex in the world, with an area of 49,606 Km^2 and 438,694 thousand inhabitants, and has the second largest buffalo population in Brazil, 377,150 thousand. Its economy is based on bubalinoculture, açaí, wood, fishing, and ecotourism (1). Buffaloes are well adapted to hot, humid climates and muddy terrain, but show signs of discomfort when exposed to direct sunlight due to their specific structural characteristics such as dark skin, small number of sweat glands/skin area and thick layer of skin epidermis (2).

It is noteworthy that systems products with the highest degree of animal comfort have added economic and ethical values, and serve a specific class of consumer market (3). Thermal stress imposes behavioral, physiological, and metabolic adaptations to reduce tension and increase animal probability survival, as well as reduce ruminant's performance and compromise animal health (4).

Regarding weather and climate conditions, in years considered as El Niño and/or La Niña, there are mechanisms that modulate atmospheric conditions and promote anomalous changes in the most diverse planet regions, including the thermal-hydro Amazon regime, affecting the monthly, quarterly, seasonal, and annual distribution of rainfall regime, and mean and extreme temperature conditions. Through the UKMO-HadCM3 model it was possible to identify that El Niño events in the 21st century will increase by 20% over the last century, and La Niña tend to intensify their frequency by five times the values observed in the twentieth century (5, 6).

In El Niño years the dry season in Amazon is intensified and this phenomenon is associated to negative anomalies of rainfall that promote river flow (7, 8). Thus, in these extreme event's years, weather conditions can intensify animal discomfort, especially when deprived of shady environments, which can be demonstrated by physiological and behavioral changes. Thus, this work aims to evaluate the effect of thermal-water variables in female buffaloes with and without direct sun exposure in intense El Niño year, through behavior responses and infrared thermography to reinforce the environmental comfort indicators, in Marajó Island, Pará, Brazil.

MATERIALS AND METHODS

During the study, meteorological data were obtained from the meteorological elements of automatic station in Soure, Pará, Brazil (**Figure 1**). The values are expressed in maximum, mean and minimum air temperature, provided by the National Institute of Meteorology (INMET). Data were also recorded with aid of thermal-hydraulic sensors (HOBO® data logger, model U12−012). They were inserted in agrometeorological mini-shelters and installed in the experimental site, such as air temperature (AT) and relative air humidity (RH). The wind speed (WS) was monitored with aid of a portable digital anemometer, model TAD - 800 (Instrutherm, São Paulo, Brazil).

Twenty Murrah buffaloes, with an average age of 24 months, average weight of 267.92 ± 28 kg, clinically healthy cyclic, non-pregnant and non-lactating females, randomly assigned to two groups (group WS – with shadow and group NS- no shadow). Animal adaptation period to adopted management was 2 days. The WS group ($n = 10$) remained on a 1 ha paddock, in the system with red jamb (*Syzygium malaccense*) and cueira (*Crescentia cujete*) native trees, which allowed about 16% of shade and was measured with tape measure in areas shaded by trees. The NS group ($n = 10$) was kept on paddocks without trees. Both groups were grazing continuously in *Brachiaria humidicola*, with access to drinking water and *ad libitum* mineral salt.

At 6:00 a.m., 10:00 a.m., 2:00 p.m., 6:00 p.m. and 10:00 p.m., for 7 days, animals were taken to the squeeze crush, where they were quickly recorded data from physiological variables that indicate thermal stress: rectal temperature (RT), respiratory rate (RR) and body surface temperature (BST). To obtain the RT, a veterinary clinical thermometer was used, with a maximum scale up to 44°C; it was inserted in the animal's rectum. RR was obtained by inspecting and counting thoracic-abdominal movements for 1 min, with help of a stopwatch. The BST was obtained with aid of an infrared thermometer (Model TD - 965 - Instrutemp, São Paulo, Brazil), activated at a distance of one meter from the measurement points on the animal: forehead, left side of the chest and left flank, using the average of the values.

After 7 days of physiological data collection, buffaloes were changed systems to eliminate animal individual effect. Then, the group that was in the WS system was transported to the NS system and vice versa. After that, animals remained in physiological adaptation again for 2 days in the new system, and then, physiological variables were collected again, for another 7 days.

With physiological variable's data, it was possible to calculate Benezra's Thermal Comfort Index to determine animal's comfort level, using the formula: $BTCI = RT/38.33 + RR/23$, where: RT is the rectal temperature (°C), and RR is the respiratory rate. Values close to two are considered to be of greater animal comfort, meaning that animals would be presenting ideal RT and RR (9).

With these climatic variable's data, Temperature and Humidity Index (THI) was calculated, proposed by Thom (1959), $THI = (0.8 \times \text{Average} + (\text{Average RH } (\%)/100) \times (\text{Average} - 14.4) + 46.4)$, where T = temperature °C and RH = relative humidity. THI values up to 70 indicate a non-stressful environment, between 71 and 78 critical, between 79 and 83 dangerous, and above 83, an emergency condition.

To more accurately assess the animal's thermal comfort situation, practical Buffalo Comfort Climatic Condition Index (BCCCI) and the practical Buffalo Environmental Comfort Index (BECI) were also calculated using the formulas: $BCCCI = 0,0571 * RU + 1.0480 * AT$ (Interpretation: up to 34.65—comfort, from 34.66 to 38.02—danger, from 38.03 to 41.39—thermal stress and above 41.39—emergency) e $BECI = 0.8854 * BST + 0.1695 * RR$ (Interpretation: up to 33.55—comfort, from 33.56 to 36.67—danger, from 36.68 to 39.79—thermal stress and above 39, 79—emergency), developed by Silva et al. (10), for buffalo in the Eastern Amazon, Brazil.

Animal's body surface temperatures were also measured using infrared thermography with a FLIR T - series T640bx camera.

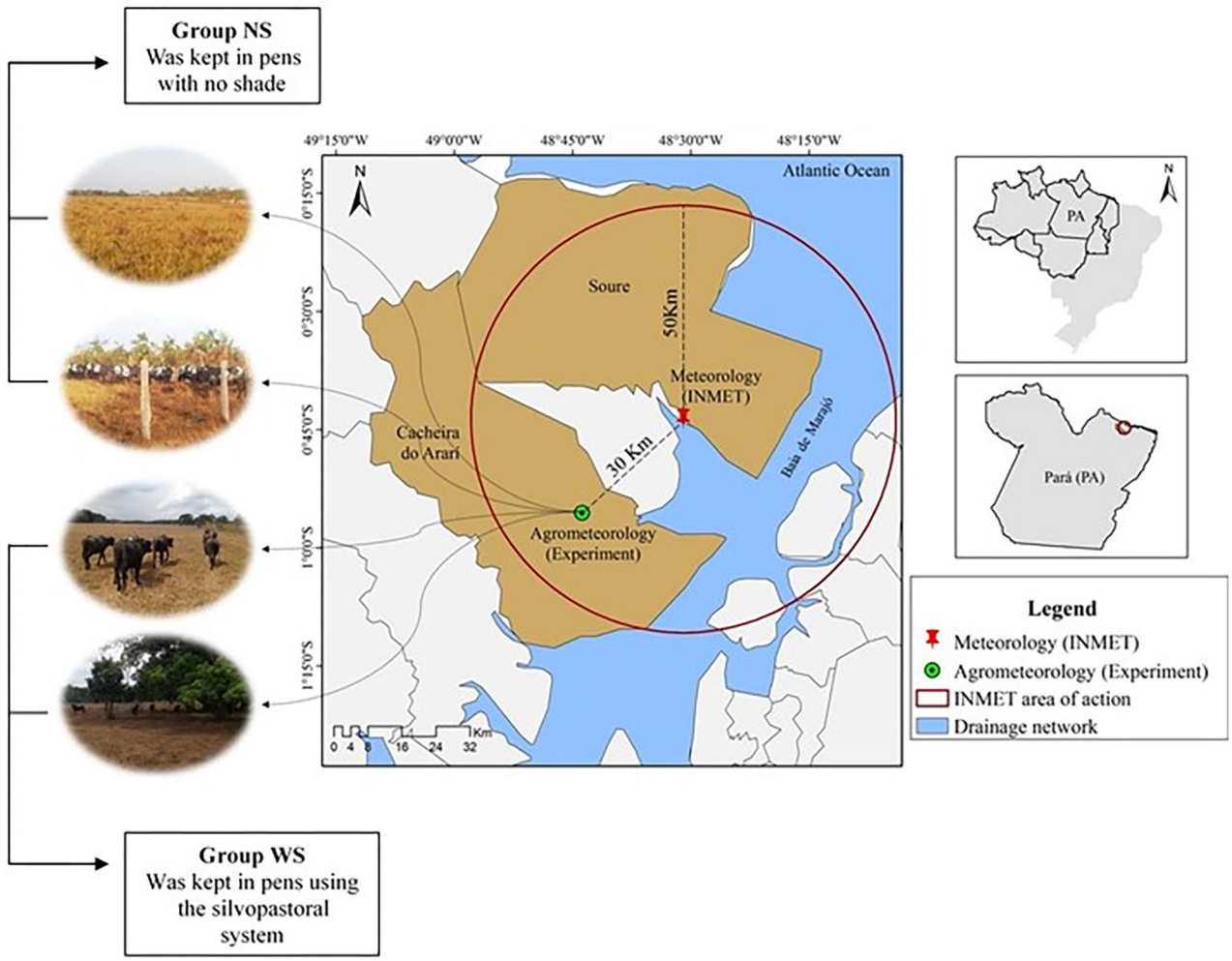

FIGURE 1 | Experimental location area in Cachoeira do Arari municipality, Marajó Island, Pará, Brazil.

Animals were evaluated at 10 a.m and 2 p.m., for 1 day, with a total number of 240 images. The camera had autofocus, temperature range −40°C, to 2,000°C, thermal sensitivity <0.03–30°C, spectral scale from 7.5 to 13 μm and optical resolution of 640 × 480 pixels, with emission of 0.98 (11). Each generated thermogram was recorded on a memory card and analyzed by the FLIR Tools software, with thermographic images containing mean, maximum and minimum temperatures, generated from four different anatomical points: orbital area (ORB), ear (EA), tail insertion (TI), and vulva (VUL) (**Figure 2**), always in that order of collections.

Thermographic image's capture was obtained with animals in crush squeeze, by a single technician, and in the same position, at a distance of one meter. Image's evaluation from ORB and EA areas was limited by a circular trace in the orbital region (12, 13), and in the TI and VUL images, rectangular shape (14).

After physiological variable's collection, animal behavior was evaluated, for 72 consecutive hours, in special files of 60 min each, divided into 12 periods of 5 min, according to the adapted methodology of Lourenço Júnior (15). Females were numbered from 1 to 20, with non-toxic ink, white in color, on the palette and posterior train, to facilitate animal's identification. For evaluation purpose, four categories of activities were considered: grazing (time spent by the animal grazing), rumination), idleness (total time spent by the animal standing or lying) and other activities (walking, defecating, drinking water, urinating and eating salt). The experimental design was completely randomized, considering five shifts of the day: morning (between 6 a.m. and 9:55 a.m.), intermediate (10 a.m. and 1:55 p.m.), afternoon (2 p.m. and 5:55 p.m.), evening (6 p.m. and 9:55 p.m.), late night 1 (10 p.m. and 1:55 a.m.) and late night 2 (2 a.m. and 5:55 a.m.).

The experimental design was completely randomized. For physiological variable's analysis, there was a crossover exchange, in which all animals underwent both treatments, in order to eliminate individual influence, lasting 7 days, and a 2days interval between them, for animal's physiological recovery and

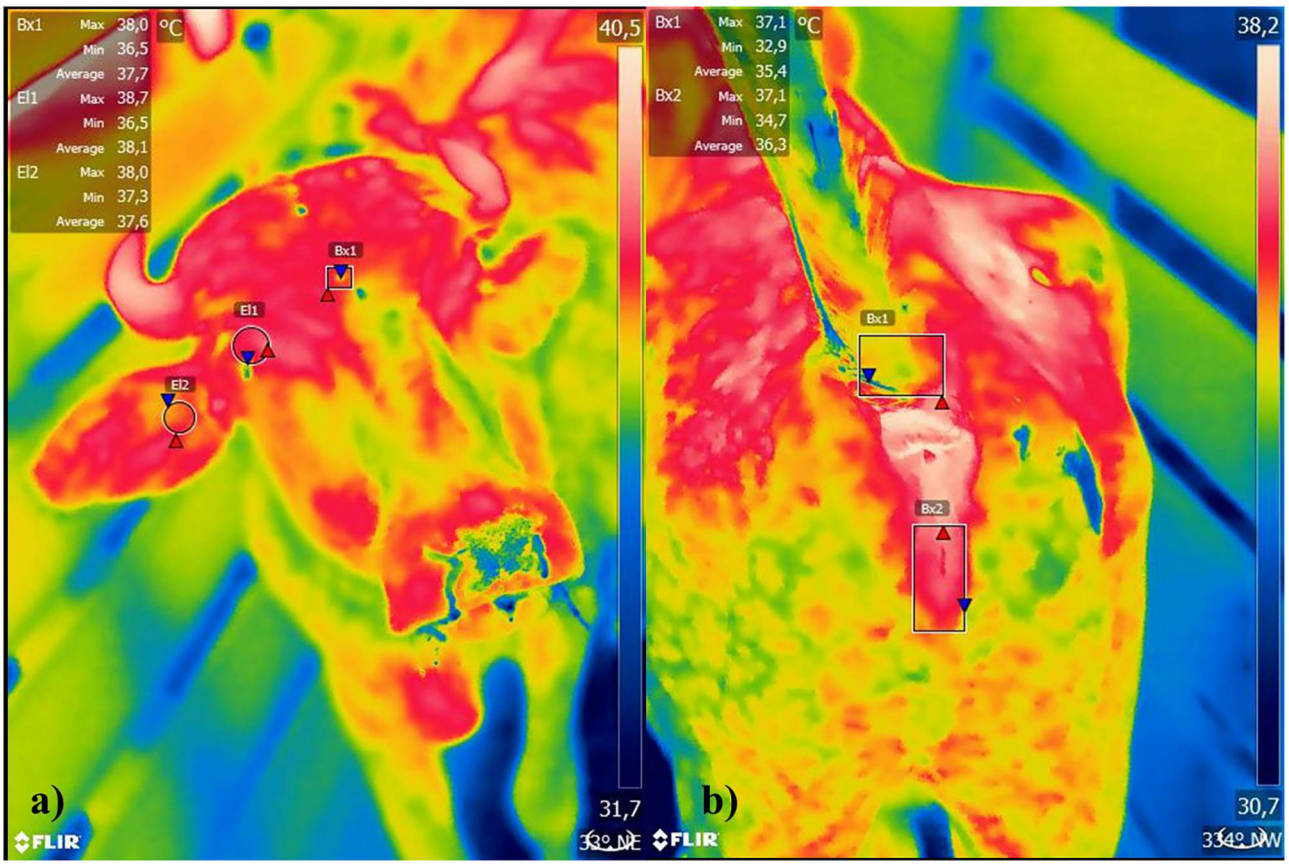

FIGURE 2 | (a) Orbital area, ear, **(b)** tail insertion, and vulva of female buffaloes reared in soure, Marajó Island, Pará, Brazil.

then switched treatment, for animal behavior assessments. For thermography, the correlation analysis between the parameters was performed using Pearson's correlation coefficient. The data for physiological (RT, BST and RR), climatic (AT, RU, and WV) and behavioral variables (grazing, ruminating, idleness and other activities) are expressed as means and standard deviations. The statistical treatment was carried out using the SAS v. 9.2 (SAS Institute, Cary, NC, USA, 2010), means being compared using the PROC GLM procedure, with Tukey's HSD test, with 5% probability.

RESULTS

The study area is within the 50 km radius of the INMET weather station (**Figure 3**). It was observed that the AT in the experimental area had behavior similar ($P > 0.05$) to the automatic surface station, in the months of October and November 2015, at times 6 a.m. and 2 p.m. The Hobo AT at 2 p.m. was higher than that found at the station, probably due to its location near the Atlantic Ocean, wind-receiving area (cyclonal). The mean AT of experimental area over days varied ($P < 0.05$) approximately 2°C in station data. On November 04 and 05, 2015, the late night hours were warmer in the experiment area when compared to the minimum in Soure. These values indicate that during this period there could be greater cloudiness, preventing heat conduction to atmosphere, making the late night warmer in the experimental area.

In the experimental area, the highest mean air temperature was at 2 p.m. in both treatments (34.58 ± 0.90 and 34.96 ± 0.71°C) was observed. There was a significant difference between the groups at 6 a.m., 10 a.m., and 6 p.m., with a variation of approximately 1°C (**Table 1**).

In the experimental area, there was a significant difference between the treatments for RH, at 6 a.m., 2 p.m., 6 p.m. and 10 p.m. (**Table 1**), except for 10 a.m., where the mean values were higher for the NS group. The highest means in both groups was at 6 a.m. (97.14 ± 6.22 - NS and 85.50 ± 3.67 - WS).

The highest WV means were found at the most critical hours of 10 a.m., 2 p.m. and 6 p.m. (**Table 2**), according to the Beaufort scale, the winds are moderate breeze at 10 a.m. and 2 p.m., and weak breeze at 6 p.m.

Regarding physiological variables, the buffaloes showed respiratory behavior changes only at 10 a.m. and 2 p.m. (**Table 3**), with higher values ($P < 0.05$) of animals from the NS Group (48.07 ± 14.06; 53.49 ± 20.48 vs. 32.76 ± 7.96; 30.30 ± 7.13 mov./min). There was also significant difference for RT (**Table 3**)

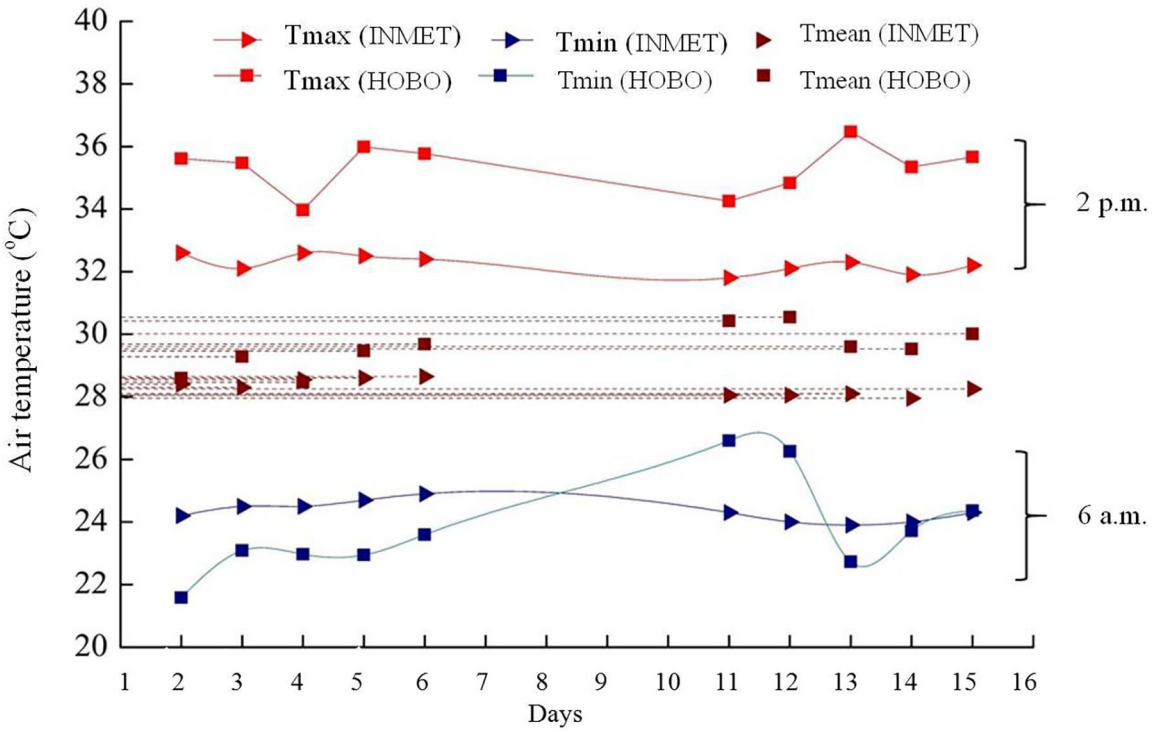

FIGURE 3 | Maximum, mean and minimum air temperature (°C) at 6 a.m. and 2 p.m. of Hobo and weather station located in soure, Marajó Island, Pará, Brazil.

TABLE 1 | Mean values and standard deviation of climate variables at different times in the experimental area, Marajó Island, Pará, Brazil.

Grupo	Air temperature (°C)				
	6 a.m.	10 a.m.	2 p.m.	6 p.m.	10 p.m.
NS	24.3 ± 1.87^{Eb}	32.6 ± 1.13^{Ba}	34.5 ± 0.90^{Aa}	29.1 ± 1.13^{Cb}	27.1 ± 0.54^{Da}
WS	25.1 ± 1.07^{Ea}	31.87 ± 1.02^{Bb}	34.9 ± 0.71^{Aa}	30.1 ± 1.07^{Ca}	27.6 ± 0.33^{Da}
	Relative humidity (%)				
NS	97.1 ± 6.22^{Aa}	62.9 ± 4.81^{Da}	54.9 ± 3.69^{Ea}	74.1 ± 6.0^{Ca}	86.4 ± 3.04^{Ba}
WS	85.5 ± 3.67^{Ab}	61.7 ± 4.40^{Ca}	49.7 ± 3.22^{Db}	63.6 ± 4.77^{Cb}	76.6 ± 1.78^{Bb}

Means within groups with equal capital letters do not differ statistically (P < 0.05). Means at each time, followed by equal lowercase letters, do not differ statistically (P < 0.05). NS, no shadow; WS, with shadow.

between treatments at 10 a.m., 2 p.m., and 6 p.m. (**Table 2**), whose values were higher ($P < 0.05$) for the animals from NS Group (38.63 ± 0, 50, 39.19 ± 0.59 and 38.65 ± 0.51°C), with the highest mean time at 2 p.m.

In this study was found that buffaloe's BST in both experimental groups showed significant changes ($P < 0.05$), only at 10 a.m., 2 p.m., and 10 p.m. (**Table 3**), with values higher than the NS Group (36.17 ± 0 97, 37.20 ± 1.20, 32.32 ± 0.55°C vs. 34.85 ± 0.83 and 35.49 ± 0.93; 31.88 ± 0.67°C), with the largest mean at 2 p.m. and lower at 6 p.m. and 10 p.m. in both treatments.

The environment, in both groups, was critical at 6 a.m., emergency at 2 p.m. and 6 p.m. in danger (**Table 4**). There was a significant difference between the 10 a.m. treatments, the NS group presented emergency conditions, and WS hazardous environment.

According to BCCCI values, from Silva et al. (10), that was developed for buffaloes raised in the climatic conditions of Eastern Amazon, Brazil; buffaloes raised at Marajó Island also are in thermal stress conditions at 2 p.m., danger at 10 a.m. and 6 p.m. and comfort 6 a.m. and 10 p.m. (**Table 4**).

BECI was significant ($P < 0.05$) only at 10 a.m. and 2 p.m. (**Table 3**), with higher values in the NS Group (40.17 ± 2.77; 42.01 ± 4.06 vs. 36.41 ± 1.68; 36.56 ± 1.67), indicating that at 10 a.m. and 2 p.m. the animals in NS group are in emergency conditions and those with access to shade are in

danger. In addition, at 6 a.m., 6 p.m. and 10 p.m. the animals in WS group are in comfort.

The BTCI was created to evaluate cattle comfort level and indicates that when values are below 2.0, animals are in greater thermal comfort (9). Buffalo BTCI in both experimental groups indicated significant difference ($P < 0.05$) only at 10 a.m. and 2 p.m. (**Table 3**); although the values in NS group (3.09 ± 0.61; 3.34 ± 0.90 vs. 2.41 ± 0.34 and 2.31 ± 0.31) were higher, both groups are higher than the considered limit for animal welfare. At other times, and in both groups, the buffaloes remained in a comfort zone. Garcia et al. (16) state that the BTCI has a moderate, positive and significant correlation with the mean air temperature, which justifies the thermal stress at higher temperatures.

Regarding thermographic image's results, Pearson correlation (**Table 5**) was significant and positive ($P < 0.01$) between RT mean and VUL, TI and ORE mean, maximum and minimum temperatures, and also, with ORB maximum temperature. VUL maximum surface temperature was more correlated (r = 0.84) when compared to eyes, tail insertion and ear.

The total time given to grazing was 518.2 min for animals from NS group and 629.5 min for WS group (**Figure 4**). Group WS intensified grazing in the afternoon and morning, while animals from group NS, the highest average was in the morning.

Rumination was more pronounced in the afternoon shift for NS group, while for shadow group it was the intermediate one, evening and late night 2.

The time spent by animals in idleness was 579.2 min in NS group and 367 min in WS group. Between treatments, there were differences ($P < 0.05$) in the afternoon, evening and late night shifts 2; NS group spent more time in idleness. The longer time is due to the shorter time involved in other activities.

In the morning and intermediate shifts, the animals from NS group obtained the highest means of time spent with other activities, probably intensifying the water intake; since in this shift the environmental situation was severe thermal discomfort. However, during late and early shift 1, WS group stayed longer in other activities.

TABLE 2 | Wind speed (ms^{-1}), mean and standard deviation, at different times, in the experimental area, Marajó Island, Pará, Brazil.

Time	Mean (ms^{-1})	Beaufort scale
6 a.m.	0.23 ± 0.71^{B}	Calm
10 a.m.	5.61 ± 3.32^{A}	Moderate breeze
2 p.m.	5.65 ± 2.90^{A}	Moderate breeze
6 p.m.	4.68 ± 2.68^{A}	Weak breeze
10 p.m.	1.71 ± 2.16^{B}	Light breeze

Means within the times, with the same capital letter, do not differ statistically (P < 0.05).

DISCUSSION

The present work took place in El Niño year, that was considered the strongest in 65 years, behind only 1982/1983 and 1997/1998 events (17). This phenomenon alters meteorological patterns of certain regions, in weather and climate conditions (18). If the mean rainfall distribution changes to higher or lower values, it may mean that flood or drought thresholds are crossed more often (19).

Differences between the groups at 6 a.m., 10 a.m., and 6 p.m. may impair animal's thermal exchange, especially in the Amazon, which presents air relative humidity. The heat lost to the environment through sensitive heat exchange occurs as a function of temperature differences between body and environment (20).

Air temperature effects are closely linked and dependent on the relative humidity level. This climatic variable is expressed as the relationship between the vapor amounts in the environment that would exist if it were saturated at the same temperature (21).

TABLE 3 | Mean values and standard deviation of physiological variables at different times in the experimental area, Marajó Island, Pará, Brazil.

Group	Respiratory rate (mov./min.)				
	6 a.m.	10 a.m.	2 p.m.	6 p.m.	10 p.m.
NS	18.6 ± 3.20^{Ca}	48.0 ± 14.06^{Ba}	53.4 ± 20.48^{Aa}	21.2 ± 4.45^{Ca}	18.4 ± 2.76^{Ca}
WS	18.5 ± 3.26^{Ba}	32.7 ± 7.96^{Ab}	30.3 ± 7.13^{Ab}	19.8 ± 3.22^{Ba}	17.7 ± 3.02^{Ba}
	Rectal temperature (°C)				
NS	37.1 ± 0.59^{Da}	38.6 ± 0.50^{Ba}	39.1 ± 0.59^{Aa}	38.6 ± 0.51^{Ba}	37.9 ± 0.50^{Ca}
WS	37.0 ± 0.57^{Da}	38.0 ± 0.36^{Bb}	38.1 ± 0.45^{Bb}	38.3 ± 0.42^{Ab}	37.7 ± 0.54^{Ca}
	Body surface temperature (°C)				
NS	31.4 ± 0.74^{Da}	36.1 ± 0.97^{Ba}	37.2 ± 1.20^{Aa}	32.4 ± 0.74^{Ca}	32.3 ± 0.55^{Ca}
WS	31.0 ± 0.75^{Da}	34.8 ± 0.83^{Bb}	35.4 ± 0.93^{Ab}	32.1 ± 0.67^{Ca}	31.8 ± 0.67^{Cb}

Means within groups with equal capital letters do not differ statistically (P < 0.05). Means at each time, followed by equal lowercase letters, do not differ statistically (P < 0.05). NS, no shadow; WS, with shadow.

TABLE 4 | Mean values and standard deviation of thermal comfort indexes, at different times, in the experimental area, Marajó Island, Pará, Brazil.

	6 a.m.	10 a.m.	2 p.m.	6 p.m.	10 p.m.
	Temperature and Humidity Index (THI)				
NS	75.4 ± 2.81^{Ea}	84.0 ± 1.01^{Ba}	85.1 ± 1.12^{Aa}	80.5 ± 0.99^{Ca}	79.0 ± 0.67^{Da}
WS	75.7 ± 1.51^{Ea}	82.6 ± 0.89^{Bb}	84.5 ± 0.89^{Aa}	80.5 ± 0.89^{Ca}	78.7 ± 0.42^{Da}
	Practical Buffalo Comfort Climatic Conditions Index (BCCCI)				
NS	31.0 ± 1.71^{Ea}	37.8 ± 0.97^{Ba}	39.3 ± 0.88^{Aa}	34.7 ± 0.89^{Ca}	33.3 ± 0.45^{Da}
WS	31.2 ± 0.98^{Ea}	36.9 ± 0.87^{Bb}	39.4 ± 0.69^{Aa}	35.2 ± 0.88^{Ca}	33.3 ± 0.29^{Da}
	Practical Buffalo Environmental Comfort Index (BECI)				
NS	30.9 ± 0.84^{Da}	40.1 ± 2.77^{Ba}	42.0 ± 4.06^{Aa}	32.3 ± 1.08^{Ca}	$31.7 \pm 0.70C^{Da}$
WS	30.6 ± 0.83^{Ca}	36.4 ± 1.68^{Ab}	36.5 ± 1.67^{Ab}	31.8 ± 0.78^{Ba}	31.2 ± 0.81^{BCa}
	Benezra's Thermal Comfort Index (BTCI)				
NS	1.7 ± 0.14^{Ca}	3.0 ± 0.61^{Ba}	3.3 ± 0.90^{Aa}	1.9 ± 0.19^{Ca}	1.7 ± 0.12^{Ca}
WS	1.7 ± 0.14^{Ba}	2.4 ± 0.34^{Ab}	2.3 ± 0.31^{Ab}	1.8 ± 0.14^{Ba}	1.7 ± 0.13^{Ba}

Means within groups with equal capital letters do not differ statistically ($P < 0.05$). Means at each time, followed by equal lowercase letters, do not differ statistically ($P < 0.05$). NS, no shadow; WS, with shadow.

TABLE 5 | Pearson correlation between rectal temperature and surface temperatures measured by infrared thermography in different buffalo anatomic areas at 10 a.m. and 2 p.m., Marajó Island, Pará, Brazil.

	RT	VUL mean	VUL min	VUL max	TI mean	TI min	TI max	EA mean	EA min	EA max	ORB max
RT	1.00										
VULmean	0.78**	1.00									
VULmin	0.48*	0.75**	1.00								
VULmax	0.84**	0.93**	0.57*	1.00							
TImean	0.69**	0.87**	0.63**	0.86**	1.00						
TImin	0.58**	0.75**	0.64**	0.73**	0.86**	1.00					
TImax	0.69**	0.84**	0.62**	0.81**	0.88**	0.81**	1.00				
EAmean	0.59**	0.61**	0.37*	0.57*	0.44*	0.35*	0.47*	1.00			
EAmin	0.52*	0.56*	0.32*	0.52*	0.39*	0.32*	0.40*	0.96**	1.00		
EAmax	0.62**	0.61**	0.37*	0.59**	0.45*	0.37*	0.49*	0.98**	0.92**	1.00	
ORBmax	0.65**	0.58**	0.11^{NS}	0.70**	0.42*	0.33*	0.48*	0.62**	0.58**	0.63**	1.00

*RT, rectal temperature; VUL mean, vulva mean temperature; VUL min, vulva minimum temperature; VUL max, vulva maximum temperature; TI mean, tail insertion mean temperature; TI min, tail insertion minimum temperature; TI max, tail insertion maximum temperature; EA mean, ear mean temperature; EA min, ear minimum temperature; EA max, ear maximum temperature; and ORB max, orbital area maximum temperature. **$P < 0001$; *$P < 0.05$; NS, not significant.*

The relative humidity importance is related to heat loss from evaporation. When the environment is very hot, the low relative humidity favors evaporative mechanisms, which occur faster and cause skin irritation and dehydration. On the other hand, high levels impair heat loss and intensify thermal stress (22). Air temperatures, between 13°C and 18°C; and relative humidity, from 60 to 70%, are suitable for rearing most animals (23). However, these conditions are not observed in the Eastern Amazon, Brazil.

Higher WV means, found at the most critical times of the day, may have contributed to heat loss by evaporation and conduction/convection, as long as air temperature is below the skin temperature. As they grow older, buffaloes suffer a hair natural thinning, avoiding a skin insulating air layer formation, which favors heat dissipation (24).

The animal's ability to withstand heat stress in tropical conditions can be assessed by respiratory rate ranging from 18 to 30 mov./min, rectal temperature from 37.4°C to 37.9°C and body surface temperature, from 25.6 to 35.5 (25).

Changes in respiratory behavior with higher NS group values can be explained due to body overheating, which stimulates the hypothalamus to send neurogenic signals that trigger panting in order to lower the temperature (26). Similar results were observed by Lourenço Júnior et al. (15), with buffalos

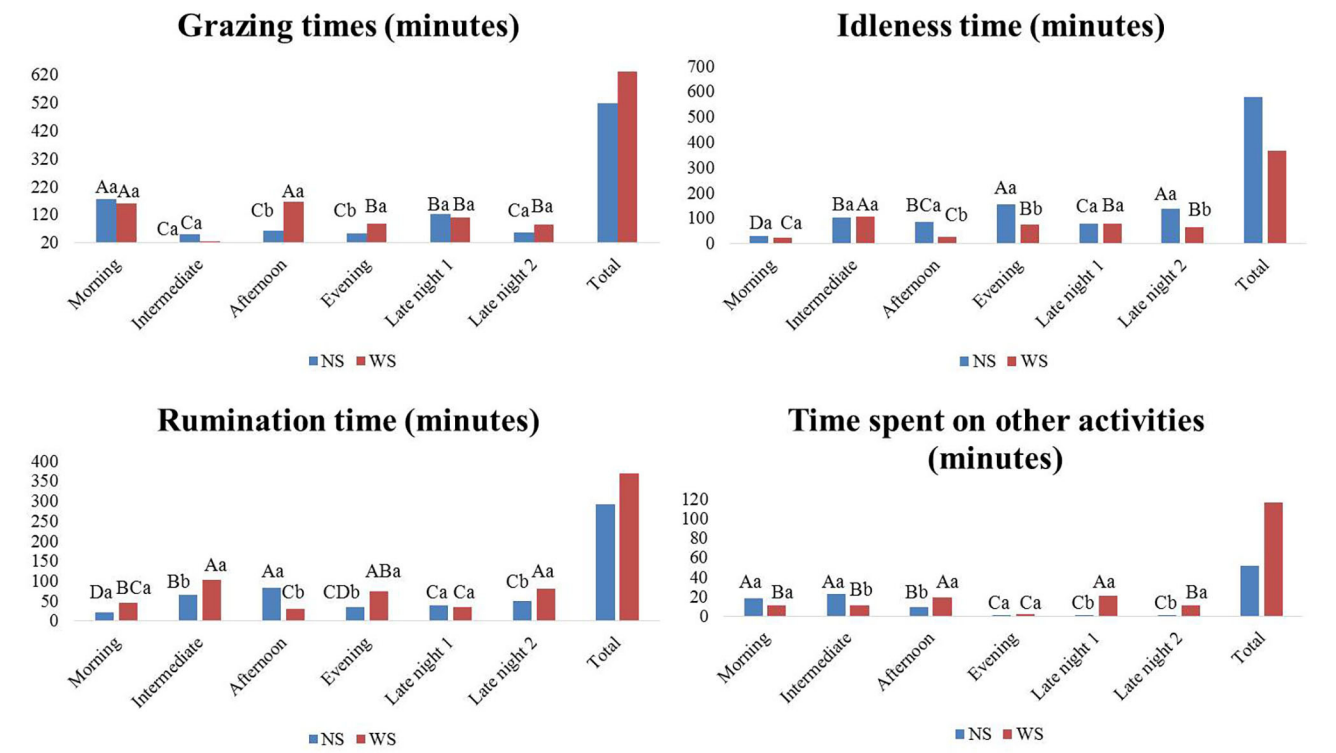

FIGURE 4 | Means of grazing time, rumination, idleness and time spent with other activities, in different shifts, in the experimental area, Marajó Island, Pará, Brazil. Shifts: morning (6:00 a.m.–9:55 a.m.), intermediate (10:00 a.m.–1:55 a.m.), afternoon (2:00 a.m.–5:55 a.m.), evening (6:00 a.m. and 9:55 a.m.), late night 1 (10:00 p.m.–1:55 a.m.) and late night 2 (2:00 a.m.–5:55 a.m.). NS, no shadow; WS, with shadow.

from Marajó island, in October and November, as the air temperature increased.

Lower RR may be related to the fact that tree shading can be effective in mitigating climate effects in tropical regions. Research in Belém, Pará, indicated that adult buffaloes raised in areas with 18 to 21% shade were protected from direct incidence of solar radiation (16). Thus, RR is a good indicator of thermal load, easy to evaluate in the field (27).

Higher values for NS group animals, with a higher average time at 2 p.m., indicate greater body heat accumulation, higher energy demand for thermolysis and, consequently, less energy available for production (16). Lourenço Júnior et al. (15) observed, in Marajó Island, values that varied from 38.7°C in the less rainy period; while Magalhães et al. (28) also observed the effect of humid tropical climate on buffaloes in the morning and afternoon shifts, 38.05 and 39.26°C, respectively.

Preoptic warming area induces blood vessel's dilatation, which consequently increases BST as blood flows (26). Buffalos still have an anatomical characteristic that differentiates them from other animals, such as thick epidermis and high melanin concentration, making them susceptible to BST elevation when exposed to direct solar radiation in tropics.

THI is considered an indicator to classify environment thermal stress degree (29). When THI exceeds 72, it negatively affects the performance of high-production animals, and can cause death in critically classified dairy cows (30). It also affects somatic cells and total bacterial count in milk (30).

All animals, regardless of rearing system, with or without shade, remained outside their comfort and welfare zone. However, it is worth noting that this index was generated for temperate conditions, making it important to evaluate these data through indices generated for tropics, such as those developed by Silva et al. (10).

Infrared thermography can be defined as a non-invasive technique of thermal mapping a body from infrared radiation normally emitted by the body surface (31). Each animal region emits a different infrared radiation, and it is interpreted as a color, according to the shade scale. Thus, certain areas of the animal's body can be related to body core temperature through the correlation of rectal temperature. In the Pearson correlation, only the maximum surface temperature for the orbital area was used, as it is considered the best representation of rectal temperature for cattle and buffalo (11–13).

Thermography imaging is a tool that is becoming increasingly popular. According to Barros et al. (11), orbital area maximum temperature has the highest correlation with buffalo and cattle rectal temperature, being the most appropriate area to evaluate the animal's thermal conditions, since it suffers interference from the ambient temperature. In addition, Brcko et al. (32) used thermography in several areas of buffalos and verified that eye and cheek could determine thermal stress because these areas

are best correlated with the animal's RT. VUL region and tail insertion also appeared to be promising body regions; however, the practical application is questionable because of invasive management for tail occlusion (13).

Accurate monitoring of grazing animal's behavior is essential to ensure health, welfare and sustainable and efficient use of grazing resources. Grazing time is the distribution and magnitude of time devoted to forage seizure; and it involves stages such as, finding and selecting the portion to be ingested, pasture cutting, mouth manipulation and rumination until to be swallowed.

The grazing interval found in this study is within the range found by Fraser and Broom (33), who report that in a period of 24 h, the animal grazes from 4 to 14 h; but there may be variation between beef and dairy animals, their origin (tropical or temperate zone) and rearing system type (free or restricted herd in the field).

Marajó Island livestock is mainly developed in dystrophic soils, resulting in lower quality forage support and productivity. In the less rainy season, the situation worsens, causing significant weight loss in ruminants, which implies longer grazing time as a way to compensate for the poor quality of forage. For Salimos et al. (34), the yield of *Brachiaria humidicola* was always above 1,200 to 1,600 kg DM/ha, regardless of the year period in Marajó Island.

Animals from WS took the opportunity to ruminate the ingested forage in the previous shift, morning, intermediate and late night 1; while group NS reduced metabolic activities, including rumination. Animals prefer to perform rumination activity in cooler periods of the day to compensate for higher internal heat production.

When exposed to high temperatures, animals will no longer eat food and they will be idleness strategically to avoid increased metabolic heat (35).

CONCLUSION

- Regardless of whether or not they have access to shade, animals experience thermal discomfort between 10 a.m. and 2 p.m. h;
- Despite environmental heat absorption throughout the day, animals had good physiological recovery, as they presented normal values of physiological variables between 6 and 10 a.m.;
- In the dry period of the year, environmental conditions of Marajó island proved to be a challenge to buffaloes thermoregulation, especially in 2015, an intense year for El Niño, when environmental temperatures were higher than normal;
- Maximum surface temperature of orbital area is the best representation of buffalo rectal temperature;
- Buffaloes kept in a shady tree system graze, ruminate and perform other activities with greater intensity than animals raised in systems without access to shade;
- Planting of trees in pasture areas is indicated to provide shade for the animals, in order to guarantee the well-being, which consequently improves their productive performance.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee from Federal Rural University of the Amazon, protocol number 054/2015 (CEUA) and 23084.013102/2015-01 (UFRA).

AUTHOR CONTRIBUTIONS

LA, WJ, and JdS: experiment design. LA, WJ, JdA, RN, and JdS: experiment perform. LA, ABa, JdS, and LM: data curation. ABa and LM: formal analysis. LA, JL, JdA, and ABe: writing-original draft. All authors edited and approved the final manuscript.

FUNDING

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) – Brasil (Finance Code 001) and Brazilian Agricultural Research Corporation–Embrapa Eastern Amazon. Also, this study received financial support for publication fee from Pró-Reitoria de Pesquisa e Pós-Graduação (PROPESP/UFPA). Finally, we would like to thank the Programa Pró-Amazônia: Biodiversidade e Sustentabilidade (Pró-Amazônia/CAPES).

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Conflict of Interest: LM was employed by the company Embrapa Eastern Amazon.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 01 December 2020
doi: 10.3389/fvets.2020.563792

Field Evaluation of the Interferon Gamma Assay for Diagnosis of Tuberculosis in Water Buffalo (*Bubalus bubalis*) Comparing Four Interpretative Criteria

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *19 May 2020*
Accepted: *05 November 2020*
Published: *01 December 2020*

Citation:
Martucciello A, Vitale N, Mazzone P, Dondo A, Archetti I, Chiavacci L, Cerrone A, Gamberale F, Schiavo L, Pacciarini ML, Boniotti MB and De Carlo E (2020) Field Evaluation of the Interferon Gamma Assay for Diagnosis of Tuberculosis in Water Buffalo (Bubalus bubalis) Comparing Four Interpretative Criteria. Front. Vet. Sci. 7:563792. doi: 10.3389/fvets.2020.563792

Bovine tuberculosis (bTB) is a worldwide zoonosis that affects many species of domestic and wild animals. *Mycobaterium bovis* is the main cause of infection in water buffalo (*Bubalus bubalis*) and bovines and is of great concern for human health and for buffalo producers in Italy. The bTB eradication programme is based on slaughterhouse surveillance and intradermal skin tests. Other *in vivo* diagnostic methods such as the interferon-gamma (IFN-γ) assay have been developed and are widely used in cattle to accelerate the elimination of bTB positive animals. The present study is the first to assess the use and performance of IFN-γ assays, which is used as an ancillary test for bTB diagnosis in water buffalo, and presents the results of a field-evaluation of the assay from 2012 to 2019 during the buffalo bTB eradication programme in Italy. The study involved 489 buffaloes with a positive result to the single intradermal tuberculin test (SITT). The IFN-γ assays and single intradermal comparative tuberculin test were used as confirmation tests. Then, a total of 458 buffaloes, reared on officially tuberculosis-free (OTF) herds, that were confirmed bTB-free for at least the last 6 years were subjected to IFN-γ testing. Furthermore, to evaluate the IFN-γ test in an OTF herd with Paratuberculosis (PTB) infection, 103 buffaloes were subjected to SITT and IFN-γ test simultaneously. Four interpretative criteria were used, and the IFN-γ test showed high levels of accuracy, with sensitivity levels between 75.3% (CI 95% 71.2–79.0%) and 98.4% (CI 95% 96.7–99.4%) and specificity levels between 94.3% (CI 95% 91.2–96.50%) and 98.5% (CI 95% 96.9–99.4%), depending on the criterion used. Finally, in the OTF herd with PTB infection, in buffalo, the IFN-γ test displayed high specificity values according to all 4 interpretative criteria, with specificity levels between 96.7% (CI 95% 88.4–99.5%) and 100% (CI 95% 96.2–100%), while SITT specificity proved unsatisfactory, with a level of 45.3% (CI 95% 35.0–55.7%). Our results showed that the IFN-γ test in the buffalo

species could reach high Sensitivity and Specificity values, and that the level of Sensitivity and Specificity could be chosen based on the interpretative criterion and the antigens used depending on the health status of the herd and the epidemiological context of the territory. The IFN-γ test and the use of different interpretative criteria proved to be useful to implement bTB diagnostic strategies in buffalo herds, with the possibility of a flexible use of the assay.

Keywords: water buffalo, tuberculosis, diagnosis, interferon-gamma (IFN-γ) test, *Mycobacterium bovis*, *Mycobacterium avium* subsp. *paratuberculosis*, ESAT6 CFP10

INTRODUCTION

Tuberculosis (TB) is a zoonosis of global importance, causing major economic losses and trade restrictions. In the year 2018, 10 million people contracted TB worldwide, of whom 1.5 million died (1). This data includes human TB caused by both *Mycobacterium tuberculosis* and *Mycobacterium bovis*; therefore, one of the objectives of the WHO is to improve the surveillance and reporting of bovine TB (bTB) in livestock and wildlife and to augment the capacity of the animal health sector to reduce the prevalence of bTB.

The first description of bTB in water buffalo (*Bubalus bubalis*) was recorded in Egypt in 1986 (2). Since then several studies reported the increasing prevalence of bTB in buffalo in many parts of the world mainly due to *M. bovis* (3–6).

About 73% of the Italian buffalo stock is bred in the Campania region in the South of Italy, according to the Italian National Livestock Database (7), where this species is of great economic importance. Indeed, buffalo rearing constitutes an important zootechnical and economic resource in a large area of central-southern Italy, where it has replaced the bovine species in the production of many dairy products, mainly the typical "buffalo mozzarella".

The prevalence and incidence rates of bTB in the Campania buffalo population were 7.30 and 3.50%, respectively, in 2018 and 13.80 and 8.9% in 2019. It should be noted that about 6,500 animals were slaughtered in 2018 and 4,600 in 2019 as a result of "test and cull" strategy adopted during bTB outbreaks. The cost to the Campania region for compensating buffalo farmers exceeded €20 million in the 2019 (data from Campania region report). It would therefore be extremely advantageous to have an accurate diagnostic strategy that could rapidly reveal bTB outbreaks during the early stage of *M. bovis* infection, in order to acquire the health status of Officially Tuberculosis-Free (OTF) territory. Improve the diagnosis of bTB is a real challenge because this disease is still causing serious economic and genetic losses as a result of the slaughter of infected animals and the depreciation of milk, which is subject to obligatory heat treatment on *M. bovis* infected farms, in a territory whose economy is closely linked to buffalo dairy products.

The oldest test for the ante mortem diagnosis of TB is the single intradermal tuberculin test (SITT), recognized by the World Organization of Animal Health and the European Commission as the screening test, used in swamp buffalo (8, 9) and in water buffalo (6). However, in buffalo species, SITT has been reported with low sensitivity (Se) and specificity (Sp) either in *Syncerus caffer* or in *B. bubalis* (9, 10).

In buffalo species, it was suggested that malnutrition leads to ineffective immune response and it could yield a false negative result, while sensitization with non-tuberculosis mycobacteria (NTM), such as *M. terrae*, *M. nonchromogenicum*, *M. vaccae*, *Mycobacterium avium* subsp. *avium,* and *M. avium* subsp. *paratuberculosis* (MAP), might reduce its Sp (11, 12). Moreover, the execution and interpretation of the intradermal test can be affected by the varying thickness of the skin in buffalo and cattle, by the black color of the buffalo skin, and the harder tissue structure. In fact, in the middle third of the cervical region, skin thickness ranges between 15 and 30 mm in buffalo vs. 5–8 mm in cattle (13).

The single intradermal comparative tuberculin test (SICTT) is used primarily as an ancillary test for positive or inconclusive reactors in the SITT [(14) 64/432/EEC]. SICTT provides a better discrimination between animals infected with *M. bovis* and those infected with *M. avium* complex or environmental mycobacteria (15–17), increasing the Sp but with a still low Se (15, 18, 19). In Brazil, it has been reported that SICTT has 71.43% Se and 82.61% Sp in water buffalo (10).

Currently, *in vitro* indirect screening tests like interferon gamma-release assays (IGRAs) are also available to detect mycobacterial infections. In human medicine, QuantiFERON®-TB Gold In-Tube test (QFT-GIT) and the T-SPOT-TB test (ELISPOT) (20) are used routinely. In veterinary medicine, in addition to SIT and SICT tests, the interferon-gamma (IFN-γ) assay, developed in Australia in the early 1990s (21) has been widely used in bovine species for the diagnosis of bTB (18, 22–27).

The World Organization of Animal Health's Terrestrial Manual included the IFN-γ assay since 1996, and the European Union adopted it as an ancillary test to the SITT since 2002 (28) to improve the detection of bTB-infected animals in a herd or in a region (29). It is believed that the IFN-γ test has the ability to provide an early detection of bTB compared to intradermal skin tests (23, 30), in fact, in several countries it is used for serial or parallel testing together with SIT or SICT tests (31, 32).

Several studies (15, 30, 33) have reported the utility of the IFN-γ test in bTB diagnosis in cattle, with a Se median value of 87.6% (range 73.0–100%) and a Sp median value of 96.6% (range: 85.0–99.6%).

However, in the literature, regarding IFN-γ test in buffalo species, scarce data are available and mainly associated with African buffaloes (*S. caffer*), (34–41).

Even though *S. caffer* and *B. bubalis* are distinguished by taxonomic classification (42) with different phenotypic traits, they belong to the same family *Bovidae* and sub-family *Bovinae,* as *Bos taurus* and *Bos indicus*. Hence, a different behavior of the cell-mediated immune response (CMI) to *M. bovis* infection is not expect.

The intradermal skin tests and IFN-γ test measure the cell-mediated immune response (CMI) to *M. bovis* infection (43). The IFN-γ test detects the cytokine produced by the T lymphocytes of infected subjects in response to stimulation with tuberculin antigens (15). The tuberculins used in both SITT and SICTT, as in the IFN-γ test, are the purified protein derivatives (PPDs) extracted from mycobacteria cultures in liquid synthetic medium. Bovine PPD (PPDB) is obtained from *M. bovis* AN5, while avian PPD (PPDA) is extracted from *M. avium* D4ER (44). In the IFN-γ test, stimulation is performed with both tuberculin antigens, PPDB and PPDA, to compare the different immune responses likewise is done in SICTT (25, 29, 45, 46).

Despite the standardization of production of PPD tuberculins and their regulation by EU (14), the estimated potency can vary between different manufacturers (15, 18, 47, 48). This could affect the performance of the IFN-γ test as described earlier (49) especially when more than one couple of PPDs were used. For this reason, we wanted to verify whether, in buffalo, the use of two couples of PPDs (Lelystad and Italian PPDB and PPDA) could give different results in the IFN-γ test and eventually improve the accuracy in different epidemiological scenarios.

The EFSA Scientific Opinion on the IFN-γ test (29) states "In infected herds (containing reactors already disclosed by tuberculin tests) the test can be applied in different ways, depending on the suspected level of infection in the herd." Therefore, to optimize the utilization of the IFN-γ test in infected herds or in OTF herds, an optimal cut-off value or an optimal interpretative criterion could be obtained with the analysis of receiver operating characteristic (ROC) curve (29, 32, 50). In this regard, several modifications to the original IFN-γ test protocol have been introduced in cattle (45, 46, 51, 52) with different cut-offs and thresholds (53) and different PPDs (49). This was done to optimize the performance and for the application of the assay in different contexts of bTB prevalence (29).

Therefore, even in buffalo, it has been useful to evaluate different interpretative criteria and cut-off values of the IFN-γ test, to adapt the assay to this animal species and in different epidemiological scenarios.

In addition, in order to improve the Sp of the IFN-γ test, specific antigens, such as 6 kDa early secretory antigenic target (ESAT-6) and 10 kDa culture filtrate protein (CFP-10) have been used during whole blood stimulation (54–57). Both EAST6 and CFP10 antigens (58, 59) are expressed in *M. bovis* but absent from NTM and *M. bovis* Bacillus Calmette Guerin. These antigens have been proposed as relevant in differentiating infected and vaccinated animal test candidates (60, 61) and used as alternative or additional antigens to the PPDs for blood stimulation in the IFN-γ assay in cattle (16, 30, 62, 63) and in African buffaloes (36).

To the best of our knowledge, there is no published literature evaluating the IFN-γ test in water buffaloes in which ESAT6/CFP10 were also used in addition to PPDs for blood stimulation.

The aim of this study was to evaluate the performance of the IFN-γ test in healthy buffaloes and naturally *M. bovis*-infected buffaloes. Therefore, we developed an IFN-γ test with combination of PPDs, a mixture of ESAT-6 and CFP-10 and four different interpretative criteria.

The final goal was to verify the use of the IFN-γ test as an ancillary test to implement bTB diagnostic strategies in buffalo herds.

MATERIALS AND METHODS

Animal Population Characteristics and Ethics Statement

The test results used for the present investigation were collected within the context of the officially ordered tuberculosis-surveillance program in accordance with Italian National [(28, 64, 65), (66): Order 9 August 2012] and Regional regulations (DD Campania n. 236/2016[1]; DD Campania n. 226/2016[2]).

Animal owners were informed of the tests carried out and all the samples were collected during the mandatory health investigations.

A total of 1,050 Mediterranean water buffaloes (*B. bubalis*) were selected in Italy from 2012 to June 2019. We evaluated the use of different PPDs and recombinant antigens and assessed the accuracy of 4 different interpretative criteria of the IFN-γ test under field conditions in herds with bTB outbreaks in OTF herds and 1 OTF herd with Paratuberculosis (PTB) infection.

The National and regional buffalo tuberculosis-surveillance program provided by Italian Ministry of Health and Campania Region [(64, 65); DD Campania n. 236/2016; DD Campania n. 226/2016] required the use of SITT as a screening test and tested positive result in OTF herds, has to be confirmed and retested. Therefore, following a positive SITT finding, the OTF herd became the "herd with suspected outbreak of TB" and sanitary restrictions were mandated, waiting for further confirmation tests such as SICTT and the IFN-γ test, after at least 42 days. The IFN-γ test was used as part of an experimental protocol authorized by the Italian Ministry of Health and the Campania region. In accordance with national and regional legislation, animals found to be positive on any one of the confirmation test were slaughtered.

A confirmed bTB outbreak was defined as a farm with positive SITT and SICTT and/or a positive IFN-γ test confirmed by isolation of *M. bovis* in at least one animal.

[1]Campania Region Executive Decree 236 of 14 October 2016. Procedures on the application of Community, national, regional and national legislation for the eradication of tuberculosis in cattle and buffaloes—amendment annexed to DD 226 of 3 October 2016.

[2]Campania Region Executive Decree 226 of 3 October 2016. Procedures on the application of Community, national, regional legislation for the eradication of tuberculosis in cattle and buffaloes.

Accuracy of Four Interpretative Criteria of the IFN-γ Test

The diagnostic Se of the IFN-γ test performed using four different interpretative criteria was calculated in a subpopulation of *M. bovis*-infected buffaloes, which were SITT and post-mortem positive. Therefore, the Se values reported in this paper refer to this subpopulation.

Hence, to assess the IFN-γ test interpretative criteria Se, we used a data set comprised of 489 bTB infected buffaloes, from 71 herds (range 1–35 animals) of confirmed bTB outbreaks in the Campania region.

Complying with the National and regional regulations mentioned above, provided by Campania Region from 2012 to June 2019, we could only include those animals that, during the activities of the regional buffalo tuberculosis-surveillance program, resulted positive to the SITT screening test in OTF herds. As stated previously, for SITT positive animals, a second access into the OFT herd, at least 42 days after the SITT, was required; official veterinarians carried out SICTT and blood sampling for IFN-γ test at the same time. Animals were deemed positive if they react to at least one of the confirmatory tests (SICTT and/or IFN-γ parallel testing). Positive animals were slaughtered in accordance with national and regional legislation, and the organs were submitted to laboratory examinations at Istituto Zooprofilattico Sperimentale del Mezzogiorno.

The performance of the IFN-γ test, particularly the Se, is usually evaluated by verifying that the animals that tested positive for IFN-γ are also positive for the isolation of *M. bovis*. In fact, culture isolation is considered the gold standard for the confirmation of *M. bovis* infection status.

However, since the Se of culture examination for *M. bovis* is low (67, 68) we decided that, in the case of infected herds, an animal was considered positive if bTB lesions had been found at the slaughterhouse and/or proved to be positive on the culture test and/or PCR.

Sp of the IFN-γ test was evaluated in a population of 458 buffaloes from 4 OTF herds during the annual SITT screening test performed in the last 6 years. The farms were located in 4 Italian regions: two farms in OTF territories in northern Italy (Piedmont, Lombardy), one in central Italy (Lazio) and one in the southern of Italy (Campania). All the herds were negative for PTB on serological testing.

A negative animal was defined as a buffalo from an OTF herd and tested negative to the SITT during the last 6 years.

Assessment of the Performance of SITT and IFN-γ on a Tuberculosis Officially Free Farm With MAP Infection

Sp of the IFN-γ test was also evaluated in a data-set of 103 buffaloes from 1 OTF herd, for the last 10 years, with PTB infection.

As NTM can interfere with bTB diagnosis (16, 41), we wanted to evaluate the performance of SITT and IFN-γ in the presence of NTM, in particular MAP, that could be present in buffalo herds (41, 69–75).

The 103 buffaloes were simultaneously tested with SITT and IFN-γ. Circulation of MAP had been confirmed by serological tests during the previous 2 years. On this farm, no buffaloes with suspect lesions of bTB had been detected at the slaughterhouse in the previous 10 years; moreover, no epidemiological link with infected farms had been established during the previous 6 years. In addition, a thorough epidemiological investigation was conducted to exclude the presence of *M. bovis* in this farm and in the neighboring farms.

Diagnostic Methods

The Intradermal Tuberculin Tests

The SITT was performed by the official/national veterinary services of the territory, in accordance with EU regulations and Italian legislation: (64, 65)—(28), O. M. 9 August 2012—and subsequent amendments. A skin fold was measured with calipers before and 72 h after the inoculation of 0.1 ml (30,000 I.U./ml) of PPDB (Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Italy).

The intradermal injection was performed by means of hypodermic needles mounted on Inj-Light syringes, at the border of the anterior and middle third of the neck, over the left shoulder of the animal, near the acromion spina scapulae (76). Results were expressed in millimeters as the difference between the two measurements, i.e., before and 72 h after the inoculation of tuberculin. The reaction was considered positive if skin thickness increased by $\geq$ 4 mm, inconclusive if >2 and <4 mm, and negative if $\leq$ 2 mm.

SICTT was also performed, but only on animals with a positive SIT screening test, from suspected-infected or infected herds.

The avian tuberculin (PPDA Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, Italy) was inoculated into the right shoulder. In accordance with National and Regional regulations, the reaction was considered positive if the difference between the PPDB and PPDA measurements was $\geq$ 4 mm, inconclusive if <4 and >1 mm, and negative if $\leq$ 1 [(28, 64, 65), O. M. 9 August 2012—and subsequent amendments).

IFN-γ Test

Heparinized blood samples were collected from each animal before the inoculation of the tuberculin and transported to the laboratory at room temperature within 8 h of collection.

Blood samples were dispensed under a laminar-flow hood in 1 ml aliquots on cell-culture plates and stimulated with two different couples of Avian and Bovine PPDs, provided by Thermo-Fisher Scientific (Lelystadt PPDs: final concentration 10 μg/ml) and by Istituto Zooprofilattico dell'Umbria e delle Marche, Italy, produced and purified as described by Corneli et al. (44) (Italian PPDs: final concentration 10 μg/ml PPDB and 5 μg/ml PPDA).

In addition, the ESAT6/CFP10 protein cocktail, produced and purified as described by Fontana et al. (77) (final concentration of each protein 4 μg/ml), was also used to stimulate blood samples.

Phosphate buffer saline (PBS), used as Nil Control Antigen (NIL), that represented the IFN-γ basal value in the single animal. A control of lymphocyte viability (pokeweed mitogen: PWM, final concentration 1 μg/ml) was also included in order to control the ability of blood cells to produce IFN-γ. In particular, PWM detects the possible presence of lymphocyte-inhibiting substances due to the illegal use of immunodepressive drugs and

TABLE 1 | IFN-γ test interpretative criteria adopted in the study.

Criteria	INTERPRETATIVE CRITERIA	
Criterion 1	PPDB-PBS ≥ 0.1 and PPDB-PPDA ≥ 0.1 = **POSITIVE**	
	PPDB-PBS < 0.1 = **NEGATIVE**	
	PPDB-PPDA < 0.1 = **NEGATIVE**	
Criterion 2	PPDB ≥ 2*PBS and (PPDB-PPDA) ≥ 0.050 = **POSITIVE**	
	PPDB ≤ 2*PBS = **NEGATIVE**	
	PPDB ≤ PPDA = **NEGATIVE**	
	PPDB ≥ 2*PBS and 0.001 ≤ (PPDB-PPDA) ≤ 0.049 = **INCONCLUSIVE**	
Criterion 3	If the basal value exceeds 0.150 OD before stimulation, the sample is considered UNSUITABLE	
	First level	PPDB and PPDA < 2*PBS = **NEGATIVE**
		PPDB ≥ 2*PBS = **BOVIS**
		PPDA ≥ 2*PBS = **AVIUM**
	If PPDB and PPDA > 2*PBS then do PPDB/PPDA	If PPDB/PPDA ≤ 0.9 = **AVIUM**
		PPDB/PPDA ≥ 1.1 = **BOVIS**
		0.9 < PPDB/PPDA <1.1 = **INCONCLUSIVE (IN)**
	Second level	If Lely PPDs = Bovis and It PPDs = Bovis then **POSITIVE**
		If Lely PPDs = Negative and It PPDs = Negative then **NEGATIVE**
		If Lely PPDs = Avium/Neg and It PPDs = Avium/Neg then **NEGATIVE**
		If Lely PPDs = IN/A/Neg and It PPDs = Bovis then **Not Discriminant (ND)**
		If Lely PPDs = Bovis and It PPDs = IN/A/Neg then **Not Discriminant (ND)**
Criterion 4	ESAT6/CFP10-PBS ≥ 0.1 = **POSITIVE**	
	ESAT6/CFP10-PBS < 0.1 = **NEGATIVE**	

The bold values are the results of IFN-γ assays according to 4 criteria
PPD, Purified Protein Derivative; PPDB, Bovine PPD; PPDA, Avian PPD; Lely, Lelystad PPDs; and It, Italian PPDs.

reveals the reduction in the immune response against various physiological or pathological conditions (57, 78).

The culture plates were incubated for 16 to 24 h at 37°C in a humidified atmosphere.

After incubation, the culture plates were centrifuged at 500 × g for 10 min at room temperature (22 ± 5°C); the culture supernatant, i.e., the plasma of each sample, was collected.

The levels of IFN-γ in culture supernatants, were measured by means of a sandwich enzyme linked immunosorbent assay (ELISA) according to the instruction of manufacturer (Bovigam, Thermo-Fisher Scientific, Schlieren, Switzerland).

The absorbance of each well was read with a 450 nm filter, and the absorbance values, expressed as optical density (OD) units, were used to calculate the results.

The quality control of ELISA assay was applied according to the instruction provided by manufacturing company which requires a range of acceptability of OD values < 0.130 for negative controls (NC) and > 0.700 for positive controls (PC). Results were excluded when the OD value for the PWM-treated sample was < 0.5 OD (45).

Four different interpretative criteria (**Table 1**) were used, in particular in the first, second, and third criterion a comparison between PPDB and PPDA was performed, applying different cut-offs to interpret the results. In the fourth criterion, the comparison was between recombinant antigens and the basal value (PBS).

Criterion 1

This criterion was the interpretation suggested by the manufacturer (Bovigam, Thermo-Fisher Scientific, Schlieren, Switzerland). It considers only PPDs supplied by Lelystad (Bovigam, Thermo-Fisher Scientific, Schlieren, Switzerland) and to define the positive sample the recommended cut-off had a net difference of PPDB–PPDA ≥ 0.1 OD if PPDB—PBS ≥ 0.1 OD.

Criterion 2

This criterion considers only PPDs supplied by Lelystad, and to define the positive sample, the recommended cut-off was the difference of PPDB–PPDA ≥ 0.05 OD if PPDB ≥ 2*PBS OD. Samples with value between 0.001 OD ≤ (PPDB-PPDA) ≤ 0.049 OD were considered inconclusive (IN).

This was the interpretative criterion used by the Italian National Reference Center for bTB, for the diagnosis of bTB in cattle infected herds alongside with SITT (29, 79, 80); the thresholds used in this criterion have also been evaluated in cattle by other authors (45, 50, 52, 81, 82).

Criterion 3

This criterion uses two couples of PPDs (Italian and Lelystad) and considers the IFN-γ tests as two separate tests, performed simultaneously.

To define the positive sample the recommended cut-off had a net ratio of PPDB/PPDA ≥ 1.1 OD if PPDB and PPDA ≥ 2*PBS OD. When ratio value was between 0.9 OD < (PPDB/PPDA) < 1.1 OD, samples were considered inconclusive.

This criterion used the ratio value obtained with stimulation of blood samples with two couples of PPDs (Lelystad and Italian). When the results of PPDs Lelystad and PPDs Italian disagree, the test was considered inconclusive and was labeled as "not discriminant" (ND). Animals with an ND result must be re-tested later after at least 42 days from the time of intradermal skin tests.

Similar to other authors (16, 32), a maximum threshold of the basal value (PBS $\leq$ 0.150 OD) has been introduced in this criterion as an additional quality control. Therefore, animals with high basal values due to pre-existing pathologies were not considered. The value of 0.150 OD was obtained by considering the mean + 7 times the standard deviation of the baseline value of 200 animals belonging to different types of rearing practices (83).

This interpretative criterion was validated at Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta laboratory and was used to eradicate bTB in Piedmont in the years from 2004 to 2016 when the region acquired the European OTF status, according to (84), and is currently used to date (85, 86).

Criterion 4

This criterion used a cocktail of ESAT6/CFP10 antigens (77) produced by the Italian National Reference Centre for Bovine Tuberculosis at Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna for the *in vitro* stimulation of heparinized blood. To define the positive sample the recommended cut-off was a net difference of ESAT6/CFP10–PBS $\geq$ 0.1 OD.

Post-mortem Diagnostic Tests

All the buffaloes found to be positive on SITT and had at least one positive result among the two confirmation tests (SICTT and IFN-γ test) were slaughtered and underwent post-mortem examination by official veterinarians to detect the presence of typical bTB lesions. Tissue samples (tonsils, retropharyngeal, mandibular, tracheobronchial, mediastinal, mesenteric, hepatic, sub-iliac, supramammary, popliteal, prescapular lymph nodes, lung, liver, and spleen) were collected for culture of *M. bovis*. The samples were transported to the laboratory and processed within 24 h or frozen at −80°C and then processed according to OIE manual protocols (17). Tissue and organs underwent culture examination and a part of the sample was subjected to direct detection of *Mycobacterium tuberculosis* complex (MTC) (87). In case of isolation of Mycobacteria, molecular, and bacteriological identification was performed as described by Boniotti et al. (87).

Statistical Analyses

The accuracy of the four IFN-γ assays interpretative criteria was evaluated on OD obtained from a total of 947 buffaloes, of which 489 buffaloes, from bTB outbreaks, tested positive at post-mortem examination and 458 bTB free buffaloes belonging to OTF herds.

The following indices were used to estimate the accuracy: Se, Sp, proportion of false positives, proportion of false negatives, area under the curve (AUC), and Youden index.

Binomial distribution was used to calculate the exact confidence limit of each proportion.

To compare the four IFN-γ assays interpretative criteria for the ROC curve analysis was performed.

Difference between the AUC for each criterion and AUC confidence limit were calculated using the package pRoc of R (88).

For the purpose of the study, Sp was defined as the proportion of samples with negative results from the expected true negative animals, while Se was defined as the proportion of samples positive results from the expected true positive animals.

Regarding criterion 2 and criterion 3, which also give indeterminate results (IN/ND), we have calculated the overall test yield which describes the probability of obtaining a positive or negative result without taking into consideration false positives or false negatives (89). Hence, we calculated the overall test yield (OTY), the negative yield (YD–), and the positive yield (YD+) as described by Simel et al. (89). The YD+ was defined as the probability of a positive result when the expected true positive animals were tested, while the YD– was the probability of a negative test result when the expected true negative animals were tested.

The agreement between the four IFN-γ assays criteria and the expected results (negative for animals belonging to OTF farm and positive at post-mortem test) was estimated on 947 animals, using Cohen's Kappa index and the McNemar-test by proc freq agree of commercial software SAS® version 4.1. A kappa value of 1 indicates perfect agreement and a value of 0 indicates no agreement beyond chance; according with McHugh (90), for the interpretation of the Kappa values, we considered a satisfied level of agreement as a kappa value > 0.9. Kappa Value between criteria were shown by heat map.

Additionally, to assess the Se of all ante-mortem diagnostic tests used in this study, in the subpopulation of *M. bovis*-infected buffaloes, which were SITT and post-mortem positive, we performed a comparison among IFN-γ test, SICTT, and $SITT_{42}$. Of these, $SITT_{42}$ is the result of the bovine PPD inoculation reaction obtained by SICTT, performed 42 days after the SITT screening.

Hence, we compared the results of the IFN-γ test obtained using four interpretative criteria and the readings of SICTT and the $SITT_{42}$. All tests were performed 42 days after the SITT screening. Se values for each test were compared using the binomial exact test.

The precision of the four IFN-γ assays criteria was also estimated in terms of reproducibility and repeatability. Reproducibility was calculated for each criterion on 32 plasma samples from 32 buffaloes: 16 positive and 16 negative from two different laboratories (Istituto Zooprofilattico Sperimentale del Mezzogiorno and Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche). The Kappa index test was used to quantify the degree of agreement between laboratories on the same sample.

Repeatability was calculated on 12 plasma samples from 12 animals, 6 of which were positive, with different OD values, 2 high, 2 medium and 2 low, and 6 negatives were randomly selected in a panel of negative samples, and three replicates were carried out by the same operator under the same test conditions. For the purpose of the study, repeatability was defined as the degree of agreement between different replicates on the same sample by the same operator, and was calculated by means of the Kappa index. Difference between proportions (Se, Sp, accuracy) was assessed by means of a binomial exact test.

TABLE 2A | Accuracy of IFN-γ assays according to 4 criteria evaluated in 947 animals.

TEST	*N*	TP	TN	FP	%FP	FN	%FN	IN/ND	SE% (CI 95%)	SP% (CI 95%)	*Y*%
Criterion 1	947	463	451	7	1.5	26	5.3		94.7 (92.3–96.5)	98.5 (98.5–96.9)	93.2
Criterion 2	947	468	313	19	5.7	10	2.1	137	97.9 (96.2–99.0)	94.3 (94.3–91.2)	92.2
Criterion 3	947	428	320	8	2.4	7	1.6	184	98.4 (96.7–99.4)	97.6 (97.6–95.3)	96.0
Criterion 4	947	368	445	13	2.8	121	24.7		75.3 (71.2–79.0)	97.2 (97.2–95.2)	72.4

N, No of animals tested; TP, true positive; TN, true negative; FP, false positive; %FP, % false positive; FN, false negative; %FN, % false negative; IN, inconclusive; ND, not discriminant; SE CI 95%, sensitivity and confidence interval 95%; SP CI 95%, specificity and confidence interval 95%; and Y, Youden's J.

TABLE 2B | Accuracy of IFN-γ assays according to 4 criteria evaluated in 947 animals.

TEST	*N*	IN/ND	Cut-off	PPV% (CI 95%)	NPV% (CI 95%)	AUC (CI 95%)
Criterion 1	947		0.1	98.50 (97.40–99.60)	94.50 (92.50–96.60)	0.966 (0.954–0.977)[a]
Criterion 2	947	137	0.05	96.10 (94.40–97.80)	96.90 (95.00–98.80)	0.961 (0.947–0.975)[a]
Criterion 3	947	184		98.20 (96.90–99.40)	97.90 (96.30–99.40)	0.98 (0.97–0.99)[a]
Criterion 4	947		0.1	96.60 (94.80–98.40)	78.60 (75.20–82.00)	0.862 (0.841–0.883)[b]

N, No of animals tested; IN, inconclusive; ND, not discriminant; PPV CI 95%, positive predictive value and confidence interval 95%; NPV CI 95%, negative predicted value and confidence interval 95%; and AUC CI 95%, AUC and confidence interval 95%. [a,b]Criterion with same letter are not statistically significant.

TABLE 3 | Results of IFN-γ assays according to Criterion 2 and Criterion 3 on 947 buffaloes tested for bovine tuberculosis.

	Expected results		Total
	Negative	Positive	
Criterion 2			
Negative	313	10	323
Inconclusive	126	11	137
Positive	19	468	487
Total	458	489	947
Criterion 3			
Negative	320	7	327
ND	130	54	184
Positive	8	428	436
Total	458	489	947

RESULTS

Accuracy of Four Interpretative Criteria of the IFN-γ Test

The accuracy of IFN-γ assays according to four interpretative criteria was assessed in 947 animals, 489 were expected to be true positive and 458 expected true negative. **Tables 2A**,**B**, **3** shows the estimates of the parameters used to calculate the accuracy.

Regarding the first criterion, 914 animals were correctly classified out of the total 947 with an observed accuracy of 96.52% (CI 95% 95.35–97.68%). The McNemar-test results were not statistically significant (p-value > 0.05). Hence, no disagreement was observed with expected results and the kappa index showed a high level of agreement (kappa 0.93, CI 95% 0.91–0.96).

Concerning the second criterion, classified 810 animals, 332 were expected true negative and 478 were expected true positive. One hundred and thirty-seven animals were classified as inconclusive (IN). Of the 137 IN animals, 11 were expected true positive and 126 were expected true negative. Criterion 2 correctly classified 781/810 animals and the observed accuracy was 96.42%, (CI 95% 95.14–97.70%). Finally, taking into account IN results, the values for OTY, YD+, and YD– were 85.53% (CI 95% 83.13–87.71%), 97.75% (CI 95% 96.01–98.87%), and 72.49% (CI 95% 68.15–76.53%), respectively. The McNemar-test results were not statistically significant (p-value > 0.05). Hence, no disagreement was observed with expected results and the kappa index showed a high level of agreement (kappa 0.93, CI 95% 0.90–0.96).

For the third criterion, 763 animals were classified, 328 were expected true negative and 435 were expected true positive. The remaining 184 animals were not discriminant (ND). Of the 184 ND animals, 54 were expected true positive and 130 were expected true negative. Related to the classification of animals, criterion 3 correctly classified 748/763 animals, the observed accuracy was 98.00%, (CI 95% 97.01–98.99%). Finally, taking into account ND results, the values for OYT, YD+, and YD– were 80.57% (CI 95% 77.90–83.04%), 88.96% (CI 95% 85.84–91.59%), and 71.62% (CI 95% 67.25–75.70%), respectively. The McNemar-test results were not statistically significant (p-value > 0.05). Hence, no disagreement was observed with expected results and the kappa index showed a high level of agreement (kappa 0.96, CI 95% 0.94–0.98).

For the fourth criterion, accuracy was assessed in 947 animals, 458 were expected true negative and 489 expected true positive. Fourth criterion correctly classified 813/947 animals, the observed accuracy was 85.90%, (CI 95% 83.68–88.12%). The McNemar-test resulted statistically significant (p-value < 0.05). Hence, no agreement was observed with expected results and the kappa index

TABLE 4 | Accuracy of IFN-γ assays according to 4 criteria evaluated on 718 animals.

TEST	*N*	TP	TN	FP	%FP	FN	%FN	SE% (CI 95%)	SP% (CI95%)	Youden's J%
Criterion 1	718	424	281	3	1.1	10	2.3	97.7 (95.8–98.9)	98.9 (96.9–98.9)	96.6
Criterion 2	718	428	276	8	2.8	6	1.4	98.6 (97.0–99.5)	97.2 (94.5–97.2)	95.8
Criterion 3	718	428	276	8	2.8	6	1.4	98.6 (97.0–99.5)	97.2 (94.5–97.2)	95.8
Criterion 4	718	345	280	4	1.4	89	20.5	79.5 (75.4–83.2)	98.6 (96.4–98.6)	78.1

N, No of animals tested; TP, true positive; TN, true negative; FP, false positive; %FP, % false positive; FN, false negative; %FN, % false negative; SE CI 95%, sensitivity and confidence interval 95%; SP CI 95%, specificity and confidence interval 95%; and Y, Youden's J.

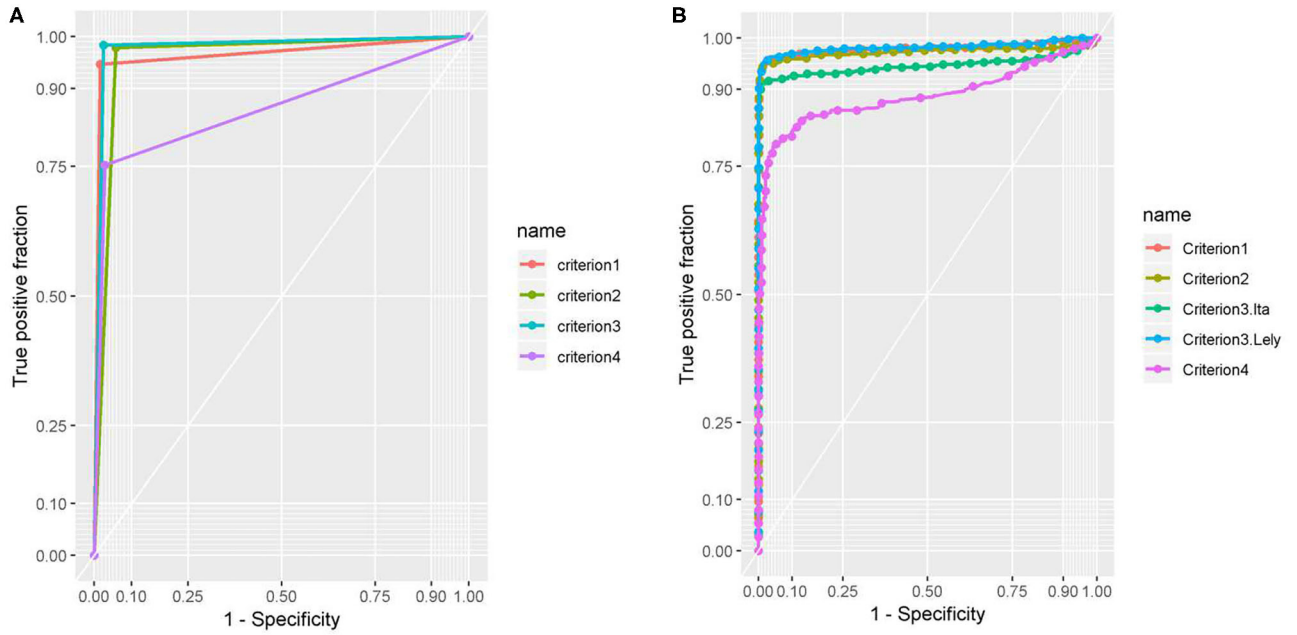

FIGURE 1 | (A) The AUC of the four criteria was estimated by binary results for each criteria. **(B)** The AUC of the four criteria was estimated by binary results for each criterion. Test 1 is OD used by criterion 1, test 2 is the OD used by criterion 2, test 3. Lel. is the OD Lelystad PPDs of criterion 3 and test 3. Ita. is the OD Italian PPDs of criterion 3. Test 4 is the OD used by criterion 4.

resulted lower than the others 3 criteria (kappa 0.72, CI 95% 0.68–0.76).

Results of ROC analysis are shown in **Table 4** and **Figure 1**. No difference resulted between AUC of first criterion, second criterion, and third criterion as DeLong's-test for two correlated ROC curves resulted statistically not significant (p-value > 0.05); while AUC of fourth criterion was different with respect to the first, second, and third criterion and the difference was statistically significant (p-value < 0.05).

The values of agreement by Kappa between criteria and between the observed and expected results are shown in **Figure 2** by heat map.

First, second, and third criterion showed higher level of agreement between them (kappa > 0.95). The Kappa agreement between the observed results of the first three criteria and the expected results was very satisfactory (Kappa > 0.93); hence, the agreement beyond chance was very high. Related to criterion 4, the kappa value ranged from 0.68 (with the second criterion) to 0.76 (with the third criterion), and these values showed an acceptable level of agreement.

The agreement between observed results of the criterion 4 and the expected results was sufficient (Kappa > 0.72).

To better compare the 4 criteria on the same number of animals, the animals that resulted IN in the second criterion and ND in the third criterion were removed from the analysis and the accuracy was evaluated in 718 animals; the results are represented in **Table 5**.

Criterion 1

Criterion 1 correctly classified 705 out of 718 animals (98.19%, CI 95% 96.92–99.03%). The McNemar-test results were not statistically significant (p-value > 0.05). Hence, no disagreement was observed with expected results and the kappa index showed a high level of agreement (kappa 0.96, CI 95% 0.94–0.98).

Criterion 2 and Criterion 3

Criterion 2 and criterion 3 showed the same results and correctly classified 704 out of 718 animals (98.05%, CI 95% 96.75–98.93%). The McNemar-test results were not statistically significant (p-value > 0.05). Hence, no disagreement was observed with

expected results and the kappa index showed a high level of agreement (kappa 0.96, CI 95% 0.94–0.98).

Criterion 4

Criterion 4 correctly classified 625 out of 718 animals (87%, CI 95% 84.37–89.42%). The McNemar-test results were statistically significant (p-value < 0.05). Hence, no agreement was observed with expected results and the kappa index resulted lower than the other 3 criteria (kappa 0.74, CI 95% 0.69–0.79).

In these animals, the performance of first, second, and third criterion, resulted the same, as the difference between criterion 1, 2, and 3 resulted statistically not significant binomial exact test p-value (>0.05).

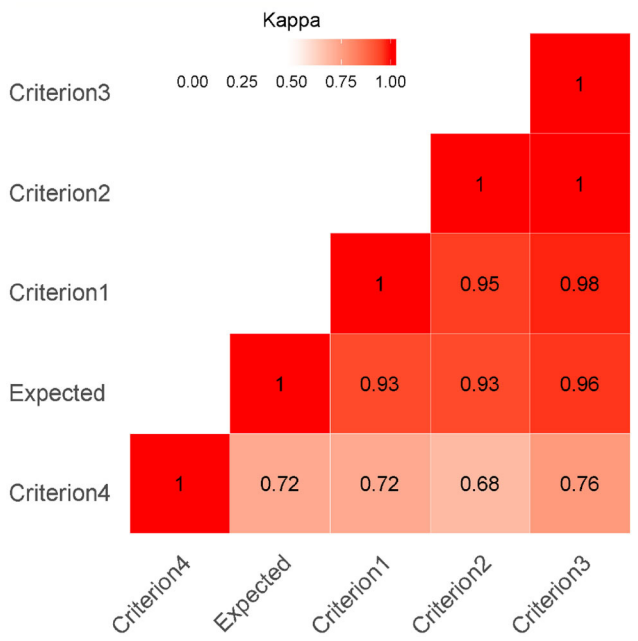

FIGURE 2 | Heat Map Kappa index as a measure of agreement was calculated on 947 buffaloes. A kappa value of 1 indicates perfect agreement and a value of 0 indicates no agreement beyond chance. The red color highlights the agreement (darker reds indicate a stronger agreement of Kappa value between criteria and expected value).

At the time of initial entry in the herds (screening test), all 489 buffaloes resulted SITT positive. All 489 buffaloes were retested, according to the Campania decree, after 42 days using SICTT and the IFN-γ test.

After 42 days, considering only the bovine PPD inoculation reaction in SICTT ($SITT_{42}$), positive animals decreased from 489 to 449 (449/489, 91.8%), as 18 animals resulted negative (18/489, 3.7%) and 22 animals (22/489, 4.5%) resulted inconclusive. As shown in **Table 5**, the Se of $SITT_{42}$ was 96.1% (CI 95%, 94.0–97.7%) and that of SICTT was 88.3% (CI 95%, 84.4–91.4%), with 41 false negative and 140 inconclusive results.

The Se of $SITT_{42}$ (96.1%) resulted higher than that by criterion 1 (94.7%), but the difference was not statistically significant (exact binomial test one-sided p-value = 0.08). The Se of $SITT_{42}$ (96.1%) resulted lower than that by criterion 2 (97.9%), and the difference was statistically significant (exact binomial test one-sided p-value < 0.02). The Se of $SITT_{42}$ (96.1%) resulted lower than that by criterion 3 (98.4%), and the difference was statistically significant (exact binomial test one-sided p-value $<$ 0.005). The Se of $SITT_{42}$ (96.1%) resulted higher than that by criterion 4 (75.3%), and the difference was statistically significant (exact binomial test one-sided p-value < 0.0001).

Repeatability and Reproducibility

For interpretative criterion 1, Kappa was 0.81 (CI 95% 0.61–1.00), indicating an almost perfect agreement between the laboratories; 3 discrepancies were observed in 32 samples. For interpretative criterion 2, Kappa was 0.93 (CI 95% 0.83–1.00), indicating an almost perfect agreement between the laboratories, only 1 disagreement was observed in 32 samples. For interpretative criterion 3, the value of K was 1.00 (CI 95% 0.99–1.00), indicating a perfect agreement between the laboratories. For criterion 4, Kappa was 0.87 (CI 95% 0.70–1.00), indicating an almost perfect agreement between the laboratories. According to the interpretation of Landis and Koch, Kappa values between 0.81 and 1 indicate an almost perfect degree of agreement; the reading of the results of IFN-γ therefore proved reproducible for each of the 4 interpretative criteria.

The Fleiss K index was calculated in 12 samples tested by the same operator at 3 different time points. The Fleiss K-value was 1.00 (CI 95% 0.67–1.00), indicating an almost perfect match.

TABLE 5 | Results obtained from 489 *M. bovis*-infected animals, tested 42 days after the SITT screening, by IFN-γ assays, $SITT_{42}$, and SICTT.

TEST	*N*	IN/ND	TP	FN	%FN	SE%	SE%CI 95%	
SITT42	489	22	449	18	3.9	96.1	94.0	97.7
SICTT	489	140	308	41	11.7	88.3	84.4	91.4
Criterion 1	489	0	463	26	5.3	94.7	92.3	96.5
Criterion 2	489	11	468	10	2.1	97.9	96.2	99.0
Criterion 3	489	54	428	7	1.6	98.4	96.7	99.4
Criterion 4	489	0	368	121	24.7	75.3	71.2	79.0

N, No of animals tested; IN, inconclusive; ND, not discriminant; TP, true positive; FN, false negative; %FN, % false negative; and SE CI 95%, sensitivity and confidence interval of 95%.

TABLE 6 | Number of animals assessed (N), True negative (TN), false positive (FP), number of IN or ND, specificity (SP) and CI 95% SP for SITT and 4 IFN-γ test criteria estimated on a sample of 95 OTF animals from a buffalo herd with MAP infection.

TEST	*N*	TN	FP	%FP	IN/ND	SP%	CI 95% SP%	
SITT	95	43	52	54.7%		45.3	35.0	55.7
Criterion 1	95	94	1	1.1%		98.9	94.3	99.9
Criterion 2	95	81	2	2.4%	12	97.6	91.6	99.7
Criterion 3	95	58	2	3.3%	35	96.7	88.5	99.6
Criterion 4	95	95	0	0.0%		100	96.2	100

Assessment of the Performance of SITT and IFN-γ on an OTF Farm Where MAP Circulated

In total, SITT was executed in 103 buffaloes, and 102 heparinized blood samples were stimulated; as 8 samples were removed from analysis owing to lack of reaction of lymphocytes against the mitogen, comparison was made in 95 animals. The results of this evaluation are shown in **Table 6**. On SITT, 43 animals tested negative, while 33 were proved as positive and 19 as inconclusive.

The Sp of SITT in this sample was 45.3% (CI 95% 35.0–55.7%), while INF-γ showed higher Sp according to all of the 4 interpretative criteria. The criterion 1 correctly classified 94 of the 95 animals. Therefore, Sp was 98.9% (CI 95% 93.4–99.9). Criterion 2 correctly classified 81 of the 95 samples; 2 animals were proved as positive and 12 IN. The Sp was 97.6% (CI 95% 91.6–99.7). Criterion 3 correctly classified 58 out of the 60 animals, as 2 animals proved positive and 35 were ND. The Sp was 96.7% (CI 95% 88.4–99.5). Finally, criterion 4 correctly classified all samples; Sp of 100% (CI 95% 96.2–100). Although the Sp varied in IFN-γ evaluation, there were no statistically significant differences observed among criteria 1, 3, and 4 (binomial exact test p-value >0.05). Moreover, between criterion 2 and 3, the confidence intervals overlapped and therefore no significant difference was observed between the Sp-values of the tests (binomial exact test p-value >0.05).

A statistically significant difference was, however, observed between IFN-γ (all criteria) and SITT (binomial exact test p-value < 0.05). Agreement between SITT and IFN-γ also proved to be scant, as the Kappa value between SITT and IFN-γ ranged between $k = 0$ for criterion 4, $k = 0.021$ for criterion 1, $k = 0.033$ for criterion 3 and $k = 0.04$ for criterion 2.

DISCUSSION

In some areas of Italy, the water buffalo is a great economic resource, as mozzarella cheese is prepared from its milk. Apart from the economic standpoint of production losses, the presence of bTB in this species is of great concern for human health. Therefore, it is necessary to implement efficient control measures to support the eradication of the disease in this species. The improvement and assessment of diagnostic techniques are the key steps, especially under field conditions, in the detection of all the infected animals present in a herd, in order to eradicate bTB.

The use of a diagnostic test as well as the set of its cut-off value to define the infectious status of an animal, requires a trade-off between the risk of keeping positive animals in the herds (Sensitivity) and the risk of slaughtering negative animals (Specificity), based on epidemiological context and local legislation (15, 29, 53).

For this reason, it is essential to provide legislators and official veterinarians with a flexible tool that, depending on the epidemiological context, allows them to decide whether to favor Se or Sp. With this aim, it was decided to combine the traditional intradermal skin tests (SITT and SICTT) with the IFN-γ test to increase the accuracy of bTB diagnostic investigation both at herd and individual level.

This study is the first to describe the evaluation of the IFN-γ assay in the diagnosis of bTB in water buffalo, comparing 4 different interpretative criteria. In particular we evaluated the accuracy of 4 interpretative criteria for the IFN-γ test in buffaloes from *M. bovis*-infected herds and OTF ones, under field conditions.

The 4 criteria chosen to evaluate the performance of the IFN-γ test have been validated in cattle and are currently used in the diagnosis of bTB in this species, in particular the first criterion is the one suggested by the manufacturer; the second is used at the Italian National Reference Center for bTB in Italy but has also been used in Spain (29, 52, 79, 80); the third is currently used at Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta laboratory and was used to eradicate bTB in Piedmont in the years from 2004 to 2016, when the region acquired the European OTF status according to Decision 2016/168 (85, 86); the fourth uses a cocktail of ESAT6/CFP10 antigens (77) for the *in vitro* stimulation of heparinized blood, it is widely used in the IFN-γ assay to improve Sp (16, 30, 62, 63).

To assess the Se of the four criteria, in bTB infected herds in SITT positive buffalo, we defined as bTB positive animals those in which were detected bTB typical lesions and/or *M. bovis* was detected by culture or PCR, introduced only recently (87).

Although the population of subjects included in the Se assessment had been selected from animals that had already tested positive in the screening SITT test, and this may have overestimated the Se of the IFN-γ test, it was still possible to fulfill the goal of this study, the comparison between the 4 criteria.

The Se of culture in the case of *M. bovis* was very low, ranging from 58.0 to 80%, on the basis of culture media and the decontamination procedure used (67, 68). This Se limitation influences the assessment of the IFN-γ test performance leading to a misclassification of data (91). Often for the IFN-γ test, the reported ranges were from 73.0 to 100% for Se and from 85.0 to

99.6% for Sp (15). This variability depends on differences in cattle populations and cut-off values adopted in the interpretation of results as well as the gold standard used for classification of bTB infection status (92). Hence, we decided to take into account other diagnostic tests to define the bTB positive buffalo.

To assess the Sp, a negative animal was defined as a buffalo from an OTF herd (at least in the last 6 years), tested negative to the SITT at the last official control.

Therefore, we defined the accuracy of the 4 interpretative criteria of the IFN-γ test in 489 expected true positive and 458 expected true negative animals, and we compered the results of the four criteria with the expected results.

The IFN-γ test parameter estimates had high Se and Sp according to all interpretative criteria (**Tables 2A**,**B**). In particular, the four interpretative criteria of the IFN-γ test showed high levels of accuracy, with Se levels ranging from 75.3% for the fourth criterion (CI 95% 71.2–79.0%) and Se 98.4% for the third criterion (CI 95% 96.7–99.4%); Sp levels were between 94.3% for the second criterion (CI 95% 91.2–96.50%) and Sp 98.5% for the first criterion (CI 95% 96.9–99.4%).

Further, we evaluated the accuracy of the criteria with the AUC analysis, the Youden index, the agreement between the observed, and the expected results from McNemar-test and the Kappa index.

For all indicators the first three criteria showed high level of accuracy, while the fourth criterion lacked Se, and showed lower level of accuracy.

Comparing the results among the criteria, the AUC of the first three criteria were very similar, while AUC of the fourth criterion resulted in a statistically significant difference (p-value < 0.05) with respect to the other criteria; same results were also observed by the agreement between the first three criteria (Kappa > 0.95).

Regarding the agreement between the observed results of the first three criteria and the expected results was very satisfactory (Kappa > 0.93) as high levels of Se and Sp were gained. Moreover, the agreement between observed results of the criterion 4 and the expected results was sufficient (Kappa > 0.72), and this was especially due to the lower values of Se (75.3%, CI 95% 71.2–79.0%).

Although the differences between the first three criteria were not statistically significant (p-value > 0.05), it is possible to observe that there are criteria capable to reveal a larger number of infected animals and criteria that, being more specific, leave many infected animals in herd (**Table 2A**).

In particular in criteria 1 and 4, respectively, the 2.75% (26/947) and 12.78% (121/947) of positive animals are not correctly identified. Therefore, these criteria would be more appropriate for situations with low bTB prevalence or in the final stages of a disease eradication plan.

On the contrary the second and the third criteria, which provide a set of inconclusive results, leave a lower number of positive animals in the herd [1.05% (10/947) and 0.74% (7/947), respectively]. Therefore, these criteria should be used in high bTB prevalence context either in herds or territories.

The same performance was observed in the data-set where the inconclusive results had been removed, and the assessment of the accuracy was performed in 718 animals (**Table 3**).

In fact, the second and third criteria introduce the possibility of animals without an outcome, the second criterion gives 14.5% (137/947) of IN results and the third criterion 19.4% (184/947) ND results. In general, these animals are more difficult to define. When evaluating all the criteria these "difficult animals" were removed and the criterion 2 and 3 had shown better values of Se (98.6% CI 95% 97.0–99.5%) in contrast to the criterion 1 (97.7% CI 95% 95.8–98.9%) and criterion 4 (79.5% CI 95% 75.4–83.2%).

However, with the second criterion, among 137 IN animals, only 11 were expected true positive and the other 126 were expected true negative. For the criterion 3, which is based on the relationship between two couples of PPDB and PPDA, among the 184 ND animals, 54 were expected true positive and 130 were expected true negative, because this criterion is the most conservative one. In fact, this criterion provides different steps and controls to define the correct sample and result; in terms of accuracy, it therefore achieves better performance (fewer false positives), but leaves more subjects without an outcome. Similar to other reports (16, 32) a maximum threshold of the basal value (PBS $\leq$ 0.150 OD) has been introduced in this criterion, an additional quality control, to make the test more "conservative" and therefore not consider suitable animals with high basal values (PBS) due to pre-existing pathologies. For these animals the official veterinarians have to repeat the blood sampling at least after 42 days from the last SITT or SICTT, this leads to longer recovery times but more accurate outcomes.

In addition, our study also suggests that using the ESAT6/CFP10 cocktail (fourth criterion), in addition to PPDs, minimizes the possibility of obtaining a false-positive result. It could therefore be a useful tool for diagnosing bTB in herds or territories in which the prevalence of bTB is low.

The four criteria also showed high levels of precision as the reproducibility and repeatability values were very satisfactory, and the tests were carried out in accredited public laboratories that have been performing the IFN-γ test for several years.

Since a major limitation to the interpretation of the *in vivo* and *in vitro* bTB assays is the cross-reactivity with responses induced by exposure to NTM, including MAP, we wanted to assess the Sp of the 4 criteria of the IFN-γ test in a particular but frequent situation a buffalo herd negative to bTB (OTF) but PTB infected for several years. In buffalo, as in cattle, infection/exposure to NTM can interfere with bTB diagnosis, because the composition of PPDs includes several antigens that can cross-react with environmental mycobacteria and this may lead to false positive reactions (93). MAP, the causative agent of PTB, is one of the most important NTM causes of false positive reactions to PPD in cattle and buffalo (11, 12, 69–75).

The data analyzed in one OTF herd MAP-infected, showed that the Sp of SITT was 45.3% (CI 95% 35.0–55.7%) lower than the values reported in cattle in the literature between 75.5 and 99.0% (15).

The lack of concordance between the Sp of the IFN-γ test (96.7% CI 95% 88.5% 89.6–100% CI95% 96.20–100%) and the Sp of SITT (45.3% CI 95% 35.0–55.7%) in the OTF herds with MAP infection was due to the use of the avian PPDs in the IFN-γ test, that were able to correctly identify

MAP-infected animals and therefore classify them as *M. bovis*-negative (15, 16, 69, 94).

In summary, our data indicate that, in buffalo, the IFN-γ assay is an excellent test and shows good accuracy which ranged from 96.42% (CI95% 95.14-97.70%) to 98.00% (CI95% 97.01-98.99%) for the three best criteria.

Since in cattle, the diagnostic Se of bTB positive animals improves when SITT is used in combination with the IFN-γ test (38, 82), we can assume that the same can also occur in buffalo. This consideration is also supported by the performance of the IFN-γ test obtained in our investigation in buffalo.

In the present study, the Se of the IFN-γ test in buffalo, which ranged from 94.70% (CI95% 92.30-96.50%) to 98.04% (CI95% 96.70-99.40%) for the best three criteria, was comparable to that indicated in cattle, with a Se estimated median of 87.6% with a range between 73 and 100% (15, 30).

With regard to Sp, our values in the buffalo ranged between 97.20% (CI95% 95.20-98.50%) to 98.50% (CI95% 96.90-99.40%) for the best three criteria, and we have achieved the best performance reported in cattle, with a Sp median value of 96.6% (range: 85.0–99.6%) (15, 30).

Moreover, taking into account that our Se values could be overestimated due to the obligate selection of animals already tested positive in the SIT screening test, our data in the *B. bubalis* displayed higher Se and Sp levels than those reported in *S. caffer* by Michael et al. (35) (92.1% Se and 68.3% Sp) and similar to the relative Se values of 100% reported by van der Heijden et al. (37).

In order to implement the results of our IFN-γ test performance evaluations, we are looking for suspected or confirmed infected buffalo herds where we can perform simultaneously IFN-γ and SITT tests on all animals of the herd.

In cattle the IFN-γ assay is incorporated into a lot of national bTB eradication programs (15, 29, 33). In particular, in epidemiological context in which the prevalence of bTB is high, but also in the extinction phase of a bTB outbreak, SITT or SICTT could be used together with the IFN-γ test. In such situations, in order to obtain higher Se, it would be useful to consider the "tests in parallel," and to classify as positive those animals that react to at least one test (37, 38, 40). By contrast, in areas where the prevalence of bTB is low, or in bTB-free herds, it would still be appropriate to use SITT or SICTT together with the IFN-γ test, it would be preferable to consider the "tests in series" and to classify as positive only animals that react to both tests, thereby improving Sp (17).

The differences in the SITT readings between the first test and the second one performed after 42 days ($SITT_{42}$), explain why the usefulness of SITT in the diagnosis of bTB in buffaloes is still debated (10, 38, 95). In fact, among the buffaloes that resulted positive to the SITT screening test, 18 animals resulted negative and 22 inconclusive to $SITT_{42}$.

These findings confirm how the SITT readings are difficult to interpret, in buffaloes, due to the tissue structure, varying thickness, and black color of the skin. Furthermore, SITT is a subjective test, because the interpretation of the reaction to PPD inoculation may vary between operators. Instead, the IFN-γ test is an objective laboratory test, which provides readings with instruments that prevent a subjective evaluation of the results (57, 78).

Several studies (18, 26, 96, 97) have shown that SICTT-negative/IFN-γ-positive animals have a 2- to 10-fold higher risk of being *M. bovis*-infected. Therefore, given the high level of risk associated with keeping SICTT-negative/IFN-γ-positive animals in an infected herd, the rapid removal of these animals appears to be the most effective measure. This reduces the potential for transmission within the herd and the future risk of recurrence of bTB infection, and to avoid a longer period of restriction or to avoid causing restriction in another herd as a result of movements.

A similar pattern of results was also obtained in our study; among the 489 buffaloes that proved to be infected with *M. bovis*, the SICTT showed low Se (88.3%; CI 95%, 84.4–91.4%) due to the highest number of inconclusive results (140/489) and 41 false-negative results (**Table 4**).

The four criteria of the IFN-γ test, among the 140 inconclusive SICTT results, detected 121 bTB positive animals (mean of the results using the four criteria) and among the 41 false-negative results, identified a mean of 36 bTB positive buffaloes.

The IFN-γ test has been proved as an objective method, as it utilizes a standard procedure and is not affected by the subjectivity of the operator, in contrast to SITT, which could be influenced by several factors that can interfere with Sp and Se (15, 30). Moreover, the IFN-γ test has a short execution time and can be repeated without time constraints. Unlike SITT, it does not interfere with the immune profile of the animal. In addition, it is not influenced by treatments with immunosuppressive drugs and is not affected, or at least is much less affected, by infection with mycobacteria other than *M. bovis*. Furthermore, its different interpretative criteria and antigens can be adopted according to the objective to be pursued and the epidemiological context (39). Our results in buffalo indicate that an IFN-γ-positive animal, especially if the test is applied in a bTB-infected herd, has a very high probability of really being infected (**Table 2B**).

Finally, countries that gained bTB eradication in cattle, including Australia, focused their attention on the herd rather than on the individual animal; these countries had considered SITT as primary screening test for bTB in herds because of its low accuracy (98), while at individual level, to maximize the detection of infected animals, they used the IFN-γ assay (17, 68).

CONCLUSIONS

In summary, our study provides new data on the Se and Sp of the IFN-γ test comparing four interpretative criteria for bTB diagnosis in water buffalo under field conditions.

Our results showed that the IFN-γ test in the buffalo species could reach high Se and Sp values, and that the level of Se and Sp could be chosen according to the interpretative criterion and the antigens used, depending on the health status of the herd and the epidemiological context of the territory.

In addition, the 4 interpretive criteria, in OTF herds with PTB, proved to be particularly useful in drastically reducing false positivity reaction for *M. bovis* compared to SITT.

Based on our results, in order to improve bTB diagnostic Se in buffalo herds, IFN-γ assays could be used in parallel with the SITT to identify the largest number of infected buffaloes in bTB outbreaks. Meanwhile, in order to improve bTB diagnostic Sp, IFN-γ assays could be used in series with SITT to limit false positive results in buffalo herds that are officially bTB-free.

Starting from the reported experience in cattle and the data of our study, in territories where bTB is still present, such as the Campania region, the use of the IFN-γ assay can support successfully the bTB eradication programme in buffalo.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The SITT and SICTT and collection of blood samples on which the IFN-tests were carried out was conducted as part of The National and Regional Buffalo TB Eradication Programme, in compliance with the EU trade (14) Council Directive 64/432/EEC, which governs the nature and frequency of testing. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

AM, ED, MP, MB, PM, and NV carried out the conceptualization of the study and performed the supervision. AM, PM, AD, IA, LS, and AC developed the methodology. NV and LC dealt with the software. AM, AD, NV, IA, FG, LS, and PM carried out the validation. NV, LC, AM, PM, MB, and MP performed the formal analysis. AM, AD, IA, LS, AC, and FG performed the investigation. AM, ED, and PM addressed the resources. NV, LC, AM, and LS performed the data care. AM, NV, PM, IA, MB, and MP prepared the original drafts for writing. AM, NV, PM, MB, MP, and ED carried out the writing—review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This research was made possible by funding from the Italian Ministry of Health for IZSME 02/2012 RC Study and validation of alternative methods for the diagnosis of bovine tuberculosis in buffalo. Testing and standardization of tests: gamma-interferon and ELISA and IZSUM 04/2011 RC Use of experimental Johnin in the gamma-interferon test for the early diagnosis of paratuberculosis in cattle and buffaloes.

ACKNOWLEDGMENTS

The authors duly acknowledge the Veterinary Services of all regions involved in the research projects for their immense help in sample collection. We also thank Dr. Monica Cagiola for providing Italian PPDs. Additionally, we would like to thank Anna Julia Heymann and Anna Donniacuo for their support in the latest revisions of the article. Finally, we would like to thank Editage (www.editage.com) for English language editing.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

REVIEW
published: 01 December 2020
doi: 10.3389/fvets.2020.570413

Bubalus bubalis: A Short Story

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The domestic buffalo (*Bubalus bubalis*), also known as water buffalo or Asian buffalo to prevent confusion with the American bison (*Bison bison*), wrongly named buffalo in North America, comprises two subspecies: the river buffalo (*B. bubalis bubalis*) and the swamp buffalo (*B. bubalis kerebau*). The swamp buffalo has a consistent phenotype and is considered as one type, even if many breeds are recognized within it; conversely, the river buffalo subspecies has many breeds. We found limited information available regarding the worldwide distribution of buffaloes. The best estimate is that 208,098,759 buffalo head are distributed in 77 countries in five continents. In this review, we presented the basic aspects of the water buffalo and unraveled the buffalo path followed from the origin of the species to its current global distribution. We reviewed several data sources to provide a better estimate of the world buffalo count and distribution.

Keywords: water buffalo, bubaline, breeds, dairy buffalo, buffalo population

OPEN ACCESS

Edited by:
Alejandra Victoria Capozzo, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

Reviewed by:
Saket Kumar Niranjan, Indian Council of Agricultural Research (ICAR), India
Rostam Abdollahi-Arpanahi, University of Florida, United States

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *07 June 2020*
Accepted: *27 October 2020*
Published: *01 December 2020*

Citation:
Minervino AHH, Zava M, Vecchio D and Borghese A (2020) Bubalus bubalis: A Short Story. Front. Vet. Sci. 7:570413. doi: 10.3389/fvets.2020.570413

THE *Bubalus* GENUS

Bovids are the largest family within the Artiodactyla order. The name bovines or Bovinae Gray, 1821 refers to a subfamily of the Bovidae family, which includes nine genera: *Bos* (cattle), *Poephagus* (yak), *Bison* (bison), *Syncerus* (African buffalo), *Boselaphus* (nilgai), *Pseudoryx* (saola), *Tetracerus* (four-horned antelope), *Tragelaphus* (kudu and relatives), and *Bubalus* (domestic buffalo) (1–3). **Figure 1** depicts the zoological classification of the Bovinae subfamily.

Bubalus, a genus of the Bovidae family, was distributed widely in Europe and Asia during the Pleistocene. Wild animals of this genus include the wild Asian buffalo (*Bubalus arnee*) that originates in northern India and lives in marshes and in the jungle. This is a very large animal, reaching a height of up to 200 cm and a weight of up to 1,000 kg; it can be either gray-black, dark gray, or dark brown and has large horns that are separated at the base (4). This species was distributed across Asia but is now considered endangered (5). The anoa, which is endemic to Indonesia, comprises two species: the lowland anoa (*B. depressicornis*) and the mountain anoa (*B. quarlesi*); however, there is some debate on whether they are the same or distinct species (6). The anoa inhabits only Indonesia, had 46 chromosomes, and is a small animal (100 cm tall) with thin and straight horns (25 cm long). It is conserved in zoos and never used for draft work or for food production and lives in mountains or in lowland forests (7). The anoa was divided in *B. depressicornis* and *B. quarlesi* based on the environment it inhabited. *Bubalus mindorensis* is found only on the island of Mindoro in the Philippines; therefore, it is also called tamaraw or Mindoro buffalo. It is a small animal (100 cm tall) with short and strong horns. It is a critically endangered species from the Philippines with only 220–300 mature animals remaining (8). **Figure 2** shows the phylogenetic tree based on the D-loop region of several species of the Bovini tribe, including the *Bubalus* genus.

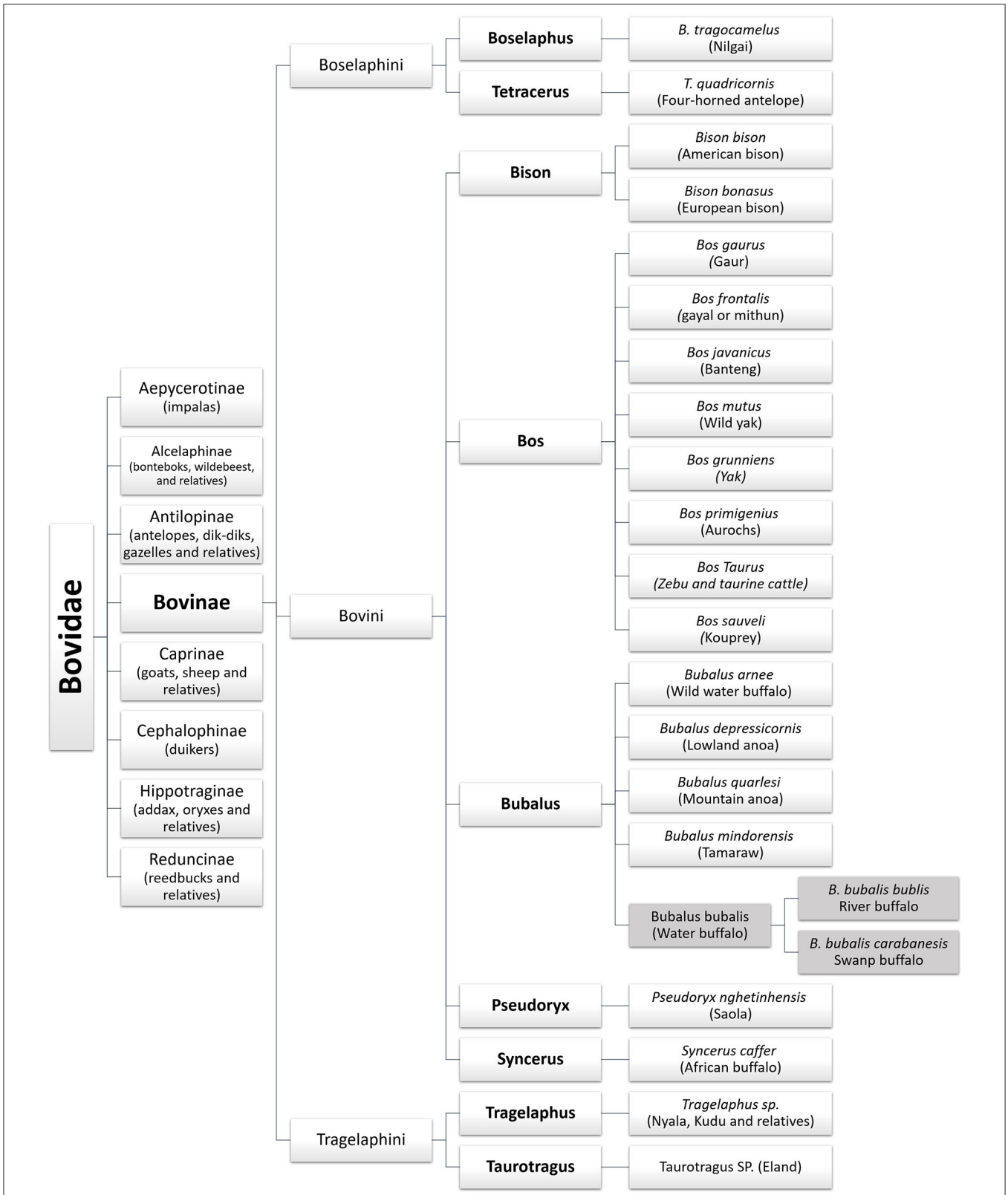

FIGURE 1 | Zoological classification of the water buffalo in the Bovinae family.

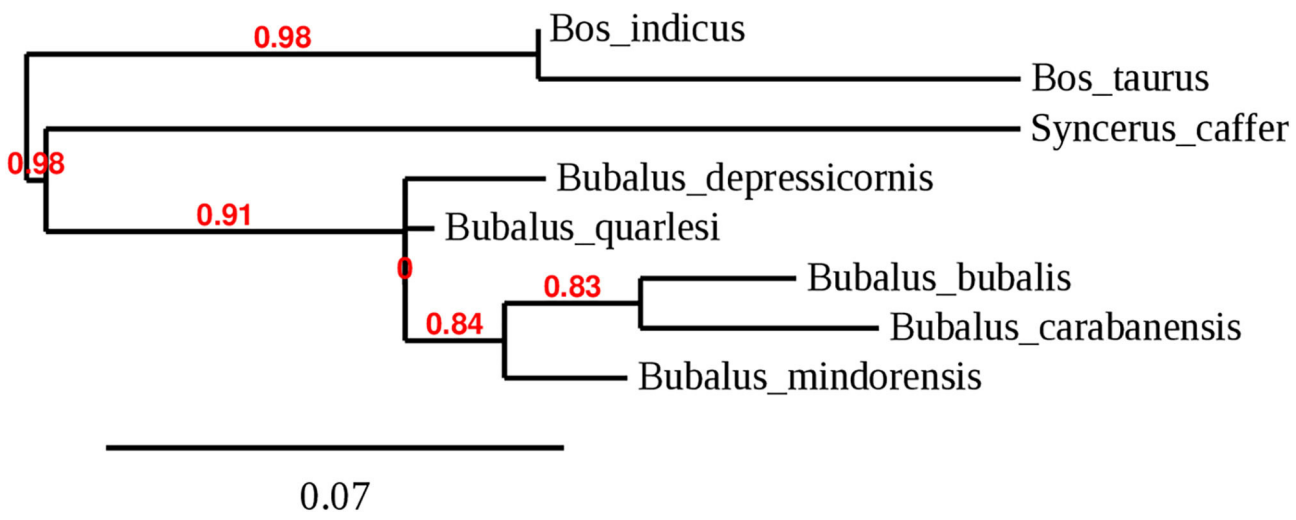

FIGURE 2 | Phylogenetic tree of the genus *Bubalus* based on D-loop region complementing with IOC and cytochrome B.

THE WATER BUFFALO

The water buffalo or Asian buffalo (*Bubalus bubalis*) is a domesticated species that can be confused by the general population with the wild American bison (*Bison bison*), incorrectly called Buffalo in North America, and occasionally, with the African Buffalo (*Syncerus caffer*), which are two unrelated species of wild animals. The water buffalo comprises two subspecies: the river buffalo (*B. bubalis bubalis*) and the swamp buffalo (*B. bubalis kerebau*), which are genetically distinct with different chromosome numbers (50 and 48, respectively), and distinct morphology, physiology, and productive and reproductive performances (9). The two subspecies are interfertile, with their progeny containing 49 chromosomes. Male crossbred progeny may sometimes present fertility problems, while female progeny exhibits longer calving intervals (10); however, this only applies in the case of further backcrossing.

The morphology of the two subspecies differs considerably. River buffaloes are usually larger than swamp buffaloes and weigh between 450 and 1,000 kg. Most breeds have curled horns and are mainly found in India, Pakistan, and some European, western Asian, and American countries. The river buffalo is primarily reared for dairy production, especially in Asia and Europe, but is also used for meat production or as a dual-purpose animal as well as for draft purposes (4, 11). Swamp buffaloes are smaller and lighter than river buffaloes and normally weigh between 325 and 450 kg. Swamp buffaloes are reared mainly for draft purposes; however, they can also yield high amounts of milk (up to 600 kg of milk per year) (11). Swamp buffaloes are predominant in Southeast Asia and Australia (12). A few animals can also be found in the north-eastern states of India (13).

Buffaloes can be affected by the same diseases and parasites as cattle; the extent to which they are affected varies drastically depending on the country, region, and production system. Buffalo breeds have varying degrees of tick resistance; however, buffaloes are infested by the louse *Haematopinus tuberculatus* that is specific to them (14). Brucellosis, tuberculosis, leptospirosis, bovine viral diarrhea, fasciolosis, foot-and-mouth disease, and protozoal infections have important economic impacts on the water buffalo industry. Regarding public health, water buffaloes are involved in the transmission of schistosomiasis to humans (15, 16). A recent report showed that buffaloes may play some part in Q fever epidemiology, as *Coxiella burnetii* was detected in buffalo milk (17).

The buffalo has a higher capacity to convert feed with poor nutritional value, and a high capacity for adaptation and survival in different environments with distinct topography, climate, and vegetation (18). The water buffalo is used as a draft animal in Asia, owing to its strength; however, it is also used as a riding animal on Marajó Island, Brazil. Water buffaloes are highly adapted to hot and wet floodplains and play an important role in the economy of many tropical and subtropical countries, as they are used for meat and milk production and as draft animals (19). Water buffaloes have relatively lower heat tolerance than other livestock species owing to their dark coat color and their inadequately dispersed sweat glands, which result in a less efficient evaporative cooling system (20). Recent molecular studies have shown that buffalo breeds historically raised in a hot climate may have developed higher heat tolerance (21). Buffaloes have higher longevity than cattle (they can surpass 30 years of age, while maintaining their reproductive capacity until they are 18–25 years old). This docile, intelligent, and curious animal (**Supplementary Video 2**) is farmed in several countries worldwide, mainly for its good quality meat (which has better nutritional composition compared to that of cattle) and its high-fat milk (11, 19, 22, 23).

WATER BUFFALO DOMESTICATION

The two distinct water buffalo subspecies (river and swamp) descended from different populations of the wild Asian water buffalo that diverged some 900,000 years before present (BP) and evolved in separate geographic regions. The wild Asian buffalo is considered the most probable ancestor of the water buffalo, and the most accepted hypothesis supports two independent domestication events from river and swamp buffalos. Recent molecular studies support that domestication started from a wild ancestor, *Swamp-like*, that was distributed across mainland Asia and has long since diverged into the existing population of *B. arnee* (9, 24). Compared with *Bos taurus* (10,000 BP), the buffalo domestication occurred recently, ∼3,000–7,000 years BP.

The domestication of river buffaloes probably occurred at ∼6,300 yr BP in north-western India, from where domesticated river buffaloes migrated west, across south-western Asia, Egypt, and Anatolia, and reached the Balkans and the Italian peninsula (25). Phylogenetic analyses and archaeological evidence suggest that the river buffalo was domesticated in an atypical and complex manner, including multiple maternal lineages, with the successive introgression of wild animals to domestic stocks (26). River buffalo populations show a weaker phylogeographic structure but higher phenotypic diversity, thereby resulting in more breeds (27). After domestication, the water buffalo was introduced in Egypt and Italy by the Arab invasion around the eighth century. Buffaloes were also introduced in the Balkans and Turkey by crusaders and by the Selgiukid invasion during the expansion of the Ottoman Empire in the fifteenth century (7).

The swamp buffalo was most likely domesticated near the border between China and Indochina at ∼3,000–7,000 years BP, spreading across Southeast Asia. Swamp buffaloes have strong phenotypic uniformity, but molecular studies show a higher diversity of maternal and paternal lineages, strong genetic differentiation affected by geography, and a lack of gene flow (27, 28). One large study with 46 buffalo populations in seven Asian countries revealed an abundant genetic diversity and clear phylogeographic structure in swamp buffalo populations (29). One exception is considered a swamp buffalo lineage from China that has a complex pattern of diversity, thereby suggesting the presence of a long-term, strong gene flow, probably due to extensive migrations of buffaloes alongside human movements (30). A recent study with buffalo whole genome showed similar genetic patterns in both cattle and buffalo breeds, an example of convergent domestication down to the same mutation but which occurred independently (31). Phylogenetic analysis revealed that Chinese swamp buffaloes could be divided into two distinct lineages, A and B. Of the two lineages, lineage A was predominant across all populations. For predominant lineage A, Southwestern buffalo populations possess the highest genetic diversity among the three hypothesized domestication centers (Southeastern, Central, and Southwestern China), suggesting that Southwestern China is the most likely location for the domestication of lineage A. However, a complex pattern of diversity is detected for lineage B, preventing the unambiguous pinpointing of the exact location of the domestication center and suggesting the presence of a long-term, strong gene flow among swamp buffalo populations caused by extensive migrations of buffaloes and frequent human movements along the Yangtze River throughout history (31).

WATER BUFFALO BREEDS

As mentioned above, the water buffalo comprises two subspecies, the swamp and the river buffalo. The swamp buffalo has a consistent phenotype and is considered as one type; however, separation in breeds still occurs, especially depending on the geographic location. Conversely, the river buffalo comprises several breeds. According to a Food and Agriculture Organization (FAO) report based on data provided by countries, there are 123 buffalo breeds, 90 in Asia alone, many of which are local breeds, with only 15 breeds reported as transboundary (32). There is a lot of variation in horn conformation. In many cases, it is one of the indicators of the degree of purity in different breeds. **Figure 3** shows photos of swamp and river buffaloes, while **Table 1** presents a list of the most important breeds and recognized populations of water buffalo.

The swamp buffalo, despite having a consistent phenotype, had somatic and physiological differences that can be noted depending on the climate and the land, owing to the total isolation of herds. The swamp buffaloes of Sulawesi that live in the jungle are completely different from the Sumatran swamp breed that lives in lagoons. The Sulawesi breed is clearly a meat animal, strong and muscular with a big head, and very appreciated in ceremonies, particularly if it is a spotted animal (**Figure 3A**), a characteristic that adds value to the animal. Sumatran buffaloes are lighter animals, with thin heads, and are mostly used for milk purposes (7).

The Carabao breed that is native to the Philippines is a swamp-type buffalo (**Figure 3B**) used as a draft animal in small-hold, land-based agriculture. Carabao buffaloes have low, wide, and heavy built bodies. Their body coloration varies from light gray to slate gray. Their horns are sickle-shaped or curved backward toward the neck (11). On the island of Marajó, Pará, Brazil, these animals are used for meat production. They are brown-gray, have white spots on their legs, are developed for work, and are suitable for meat production, while their milk production capacity is usually poor (33).

The Chinese buffalo is a swamp-type buffalo, used mostly for draft work, and, depending on the region, comprises a diverse array of breeds, which amount to 18 local breeds. The most important of these breeds are the Fuling breed (**Figure 3C**) living in the Sichuan mountains, and the Binhu breed, living in Hunan province; these are the breeds with the highest populations, which amount to more than 400,000 head each. Other breeds include the Fuzhong and Xilin breeds from Guanxi province, Enshi breed from Hubei, Dongliu breed from Anhui province, and the Shanghai breed. Swamp breeds in China are mainly used for draft purposes as they are very efficient in the marshlands, particularly in rice fields, and have limited milk production capacity (10, 28).

Swamp subspecies are also dominant in Southeast Asian countries such as Malaysia, Laos, Cambodia, Vietnam, Myanmar, and Thailand. In these countries, the buffalo is an important part of the crop production system, as it is the main draft power

FIGURE 3 | Buffalo breeds worldwide. **(A)** Sulawesi breed spotted animal (Indonesia); **(B)** the Carabao breed from Philippines; **(C)** Fuling breed in China; **(D)** Murrah buffaloes herd from São Paulo, Brazil; **(E)** Nili-Ravi breed at Pakistan; **(F)** Mediterranean Italy breed raised in Mozambique; **(G)** Jaffarabadi bull in Brazil; **(H)** Buffalypso breed from Trinidad and Tobago. Source: Castro, S.R., Borghese (7).

TABLE 1 | Breeds or recognized population of water buffalo worldwide.

Breed or recognized population	Subspecies	Local	Purpose
Anatolian	River	Turkey	Milk and meat
Azeri or Caucasian	River	Azerbaijan, Georgia Armenia, Iran	Milk and meat
Azikheli	River	Khwazakhela, Swat, Pakistan	Milk
Bangladeshi breed	River	West Bangladesh	Milk and draft
Bangladeshi Surti	River	Central Bangladesh	Milk
Bhadawari	River	Uttar Pradesh, India	Milk and draft
Binhu	Swamp	Hunan, China	Draft
Buffalypso	River	Trinidad and Tobago, Cuba	Selected for meat and draft
Carpathian	River	Transylvania, Romania	Milk and meat
Dongliu	Swamp	Anhui, China	Draft
Egyptian	River	Egypt	Milk and meat
Enshi	Swamp	Hubei, China	Draft
Fuling	Swamp	Sichuan, China	Draft
Fuzhong	Swamp	Guanxi, China	Draft
Jafarabadi	River	Gujarat, India and Brazil	Milk and meat
Jerangi	River	Andhra Pradesh, India	Draft
Kalahandi	River	Orissa, India	Draft and milk
Kerbau Papangan	Swamp	South Sumatra marshlands	Milk and meat
Khuzestani	River	Iraq and Iran	Milk and meat
Kundi	River	Sindh, South Pakistan	Milk
Lime	River	Nepal mountains and river valleys	Triple aptitude
Manda	River	Andhra Pradesh, India	Milk and draft
Mazandarani	River	Iran	Milk and meat
Mediterranean	River	East Europe, Macedonia, Greece, Serbia, Albania	Triple aptitude
Mediterranean Italian	River	Italy and export (live animals and semen)	Selected for mozzarella and processing industry
Meshana	River	Gujarat, India	Milk
Mesopotamian	River	Iraqi marshlands	Milk and meat
Murrah	River	India and export (live animals and semen)	Selected for milk
Murrah, Bulgarian	River	Bulgaria	Milk
Nagpuri	River	Madhya Pradesh, India	Milk and meat
Nili-Ravi	River	Pakistan and Punjab, India	Selected for milk
Original Far East Buffalo	Swamp	Thailand, Vietnam, Laos, Cambodia, Myanmar, Malaysia	Triple aptitude, family animal, rice fields
Pandharpuri	River	Maharashtra, India	Milk
Parkote	River	Nepal mountains and river valleys	Triple aptitude
Philippines Carabao	Swamp	Philippines rice fields	Triple aptitude
Primitive Bangladeshi	Swamp	East Bangladesh	Draft and meat
Sambalpuri	River	Madhya Pradesh, India	Milk
Shanghai	Swamp	Shanghai, China	Draft
South Kanara	River	Mangalore west coast	Draft
Sulawesi spotted	Swamp	TanaToraja Sulawesi, Indonesia	Sacrificed during ceremonies
Surti	River	Gujarat and Rajasthan, India	Milk
Tarai	River	Uttar and Madhya Pradesh, India	Milk and meat
Toda	River	Madras, India	Milk and for ceremonies

source for land preparation, supplies fresh organic manure for cultivation, and is highly adapted to living in different habitats. Australia imported swamp animals from Asia as the management systems in the country are extensive (11, 34).

The river subspecies breeds have been named according to Mason (35). There are three international river breeds that are widely distributed worldwide, owing to their high genetic potential for milk production: Murrah, Nili-Ravi, and the Italian Mediterranean (36).

Murrah is the most represented and well-known buffalo breed in the world. It is selected for milk production in northwest India and is known for its curled horns (**Figure 3D**). Its name is a

Hindu word that means spiral and derives from the shape of its horns. Its skin and hair color is jet black. White spots are not accepted, except at the end of the tail. These are massive and stocky animals with heavy bones. Their horns are short and tightly curled. This breed originated in the center of Haryana and spread across India. It has been exported to several countries worldwide, such as Brazil, Bulgaria, and East Asian countries (10, 37). In Bulgaria, the only buffalo breed is the Bulgarian Murrah, which resulted from crossbreeding the Indian Murrah and the local Mediterranean breed. Many animals have been exported from Bulgaria to neighboring countries, such as Romania and Germany, and to distant countries, such as Brazil and Venezuela. Murrah buffaloes were exported to China and Indonesia to increase milk availability, as the local breeds are of the swamp type with low milk production capacities; the same applies for many Asian countries, in which the original buffalo population is of the swamp type (19, 37).

The second most represented breed in the world is the Nili-Ravi (**Figure 3E**). This is the most important livestock breed in Pakistan with a head count higher than 10,000,000 in Punjab, while it is also present in India (38). This breed resembles the Murrah breed but has white markings on its extremities and walled eyes; its horns are less curled than those of the Murrah breed. The skin color of the breed is generally black, but there are some albino animals, brown, spotted, or with clear eyes (38, 39). Nili-Ravi is the most important dairy buffalo breed in Asia and is selected for this purpose, with its milk yield reaching 3,050 kg (40). The Kundi breed is widespread in the Sindh region in south Pakistan (10). The Azikheli buffalo is an undocumented buffalo breed from Khwazakhela, Swat, Pakistan. It has a small body size, brown-colored coat, and horns lying flat, bending laterally, and directed backward and slightly upward without twisting (41).

The Mediterranean buffalo descended from the river buffalo introduced to Europe from India during the Arab occupation of the eighth century in Sicily and in the south of Italy. The Italian breed is now classified as Italian Mediterranean breed (**Figure 3F**) as it was selected for 60 years for milk purposes and is clearly a dairy breed. This breed was later exported to several countries in America and Asia, with a large export of Mediterranean semen from Italy. Buffaloes were also introduced in east Europe by crusaders in the twelfth century and later with the Turkish invasion of the Ottoman Empire (fifteenth century). These breeds are different from the Italian Mediterranean breed. Balkan breeds such as the Carpathian or the Macedonian are draft animals, used in carriages, and they are smaller in size and with low milk yield capacity (36).

European Mediterranean animals have black, brown, and dark gray coats, while Italian Mediterranean animals are exclusively black. Their horns are flat at the bottom and face backward, and their points face upward and inward. The Mediterranean is a compact breed with a deep and wide chest, but short back and rump. The udder is medium-sized with squarely placed quarters and halves and with cylindrical teats (10, 36). The breed's size, weight, and productivity vary greatly depending on the genetics, environment, and management system. The average daily milk yield varies widely and depends on factors such as genetics and the feeding system. It can range from 3 to 4 kg milk/day for poorly fed animals up to 15 kg milk/day in intensive production systems (10). The Italian Mediterranean breed has high milk yield and quality, as it was selected for the creation of a dairy breed specialized for the cheese industry, with a genealogical book established in 1980, and the National Association of Buffalo Breeders (ANASB) established in 1979. The use of the milk data recorded for females registered on the genealogical book, the application of several cycles of progeny testing, and the spread of artificial insemination with the use of semen of proven high genetic value created an improved dairy breed, with many females producing more than 5,000 kg milk/270 days of lactation, to a maximum of 5,600 kg with 8.3 fat and 4.6 protein, as the selection goal was not the total milk yield but mozzarella production (7).

The Jafarabadi breed (**Figure 3G**) is originally from Gujarat, India. It is a large animal with a massive and long-barreled conformation and black coat. Its horns are long, heavy, begin at the base of the head, face downward, and then curve. The Jararabadi breed is of particular importance in India, with animals being exported to Brazil. In Brazil, the Jafarabadi breed has two varieties: the Gir, which is of medium size and the most widespread variety, and the Palitana, a large breed used in crossing.

The milk yield of the Jafarabadi is good; however, this is also a large animal with high muscularity. Therefore, this breed was selected by many countries in the Americas as a meat-purpose breed. It is possible to find pure Jafarabadi animals or their descendants in Brazil, Colombia, and other South American countries, where this breed showed great adaptability to marshlands and other environments, and a high affinity for meat production. Additionally, the Jafarabadi was the basis for the creation of the Buffalypso breed in Trinidad and Tobago and in Cuba (7).

The Buffalypso is the typical buffalo breed of Trinidad and Tobago (**Figure 3H**) derived from the Jafarabadi breed imported in Trinidad in 1905. In 1948, Steve Bennet crossed Bhadwari bulls with Jafarabadi cows to create the Buffalypso breed (Buffalo × Calypso, a form of popular Caribbean music), with the purpose of obtaining a meat breed, which is also useful as a draft animal. Subsequently, the Buffalypso was crossed with the Murrah, Surti, Nili, and Nagpuri breeds. It is now also used for milk production (10, 33). The highest Buffalypso head count is in Cuba and Trinidad and Tobago; however, the breed can also be found in several countries such as Venezuela, Costa Rica, Guatemala, the Republic of Honduras, Nicaragua, Brazil, Panama, Mexico, Colombia, USA, and even Taiwan. Historically, Buffalypso is the only breed selected exclusively for meat, as it is a very muscular animal with developed back and rump, brown-colored, and sold in the American market as beef.

Other river breeds in India include the Surti breed in north Gujarat, one of the most important breeds in the Rajasthan and Gujarat regions, with a population higher than 500,000 buffalo head. These animals have two white chevrons on their chest, and their horns are flat and sickle-shaped, directed downward and backward, and then turn upward at the tip to form a hook (13, 42). The Tarai breed amounts

to ∼1,000,000 buffalo head. These animals have long and flat horns with coils bending backward and upward. This breed is raised in Uttar Pradesh and Madhya Pradesh (4, 10). Other breeds of ∼300,000–400,000 buffalo head are the Mehsana (its characteristics are intermediate between the Surti and Murrah breeds), reared between the Mahi and Sabarmati rivers in Gujarat, and Nagpuri, a local breed improved by selection from Indian breeds that have long (50–65 cm) and flat-curved horns (4, 10).

Other breeds with smaller populations are the Bhadawari raised in Uttar Pradesh and Madhya Pradesh, the Jerangi (a small animal) and Manda (each amounting to 100,000 buffalo head), which are breeds raised along the border of Orissa with Andhra Pradesh, Sambalpuri from Bilaspur in Madhya Pradesh, and Toda, which is a large breed raised in the Nilgiri hills of Madras (7). Additionally, there are Pandharpuri, Kalahandi, and South Kanara River buffalo breeds in India.

In Nepal, the Lime breed is believed to have descended from the ancestral wild buffalo, possibly with river-type introgression, and became domesticated in the country (43). The Lime buffalo is found in the mountains, high hills, and hill river valleys in Nepal with a herd population of ∼700,000 head, which accounts for ∼35% of the total indigenous buffalo population. This is a small-sized breed, with small sickle-shaped horns that are curved toward the neck, and light brown in color with chevrons of gray/white hair below the jaw and around the brisket (7).

The Parkote buffalo is a typical animal of the mid-hills and river valleys in Nepal. However, owing to the traditional crossbreeding with the Lime and, more recently, the Murrah breeds, their pure breed population estimated at 500,000 head is now declining. The Parkote have dark-colored coats, black skin, black muzzles, and no markings on their legs. These animals are medium-sized, with sword-shaped horns directed laterally or toward the back (10, 35).

The native buffaloes in the western part of Bangladesh are of the river type, known as the Bangladeshi breed, and account for ∼45% of the total population. The coat color of these animals is normally black, while they have curly horns. Few individuals have spots in their tail switch, and some are brown-colored or have hanging horns. Their average milk yield is ∼620 kg in 270 days of lactation. Male buffaloes are used for draft and females for dairy purposes (44).

The Anatolian buffalo that descended from the migratory Indian buffalo (seventh century) has been raised in Turkey for centuries. These animals are black-colored and have long hair, with variations in their tail lengths and frequent white switches. The Anatolian breed has an estimated population of 122,000 and is raised predominately in the Black Sea region, north of middle Anatolia, Thrace, Hatay, Mus, Kars, Diyarbakir, Afyon, and Sivas (45).

The Azeri or Caucasian breed originated in the Indo valley and descended from the Indian buffalo. Archeological evidence suggests that buffaloes were raised in Iran (Lorestan region), around the ninth century B.C., as buffalo heads have been found engraved on a bronze stick from the same period (11, 46). This is a black-colored breed, with short horns growing backward and a population size of ∼600,000 head. It is found in Iran, Azerbaijan, and along the Caspian Sea; it was widespread in Georgia and Armenia until 1940, but then its numbers declined (47).

The Khuzestan buffalo (sometimes called Khuzestani) is a breed amounting to hundreds of thousands head raised in Iraq and Iran. They are large animals, probably the biggest buffalo breed in the Near East, with short horns pointing upward and forming a ring at the end (7, 46). Raised mostly for milk purposes, the average milk production of these animals is ∼1,950 kg of milk within 210–240 days of lactation, with a reported average of 8.6 kg/head/day for Khuzestan buffaloes (48). The Azeri and Khuzestan buffaloes, the most common indigenous breeds of Iran, present genomic regions associated with adaptation to different environments resulting from divergent selection in different regions (21, 49). In Iran, there is also the Mazandarani breed raised mainly in the Mazandran province (in the north region of the county) with 4,000 individuals (47).

The Egyptian breed was introduced to Egypt from India, Iran, and Iraq, probably around the seventh century B.C. The differences between the Egyptian buffalo types depend only on the environment. This breed is very important in the dairy industry in Egypt, as it produces 45% of the milk consumed in the country (50). These animals are blackish gray in color, with long and narrow heads, and horns varying in format (from lyre to sword-shaped) (7).

WATER BUFFALO HERD

The most robust information regarding the buffalo population is available by the FAO and is collected exclusively from official governmental institution records (51). When there is an absence of official records, the FAO statistics do not count the buffalo herds in that country, which produces a lack of consolidated information regarding the number of countries with buffaloes and the number of buffalo herds worldwide. For instance, FAO statistics indicate that only three countries in the Americas have buffalo populations (Brazil, Trinidad and Tobago, and Suriname), excluding two large buffalo herds in America, Colombia, and Venezuela. Therefore, we conducted an extensive literature review to provide a more accurate count of buffalo herds and to establish the buffalo distribution in the different continents.

The worldwide water buffalo population reported by the FAO in 2017 was ∼201 million head with an expansion to 206.6 million in 2018, showing an increase of 2.8% in 1 year. The FAO data includes 43 countries with buffalo populations but did not present an actual herd number in two of those listed countries, Australia and Singapore. There are some reports of buffaloes distributed in 129 countries, first mentioned by Iamartino et al. (52) and replicated by Rahmaninia et al. (47), but this information is inaccurate. The number 129 represented the total number of countries involved in the FAO report and does not necessarily mean that buffaloes exist in each of those countries. Thus, taking as a starting point these FAO data from 2018 and researching recent literature, we retrieved a list of 77 countries that have buffalo herds, with an estimation of 208,098,759 head. **Table 2** presents our aggregate

TABLE 2 | Current data on buffalo population worldwide.

Region/country	Water buffalo population†
Worldwide	**208,098,759**
Africa (%)	**3,509,256 (1.69%)**
Egypt	3,506,061
Mauritius	25
Mozambique	1,000
South Africa	170
Tanzania	2,000
Americas (%)	**2,562,711 (1.23%)**
Argentina	121,276
Belize	632
Bolivia	35,000
Brazil	1,390,066
Canada	1,120
Chile	100
Colombia	336,417
Costa Rica	20,000
Cuba	60,000
Ecuador	10,000
El Salvador	250
Guatemala	5,000
Honduras	1,500
Mexico	45,000
Nicaragua	800
Panama	4,000
Paraguay	15,000
Peru	1,500
Suriname	905
Trinidad and Tobago	6,145
United States of America	7,000
Uruguay	1,000
Venezuela	500,000
Asia (%)	**201,428,230 (96.79%)**
Armenia	717
Azerbaijan	176,195
Bangladesh	1,485,000
Bhutan	531
Brunei	2,319
Cambodia	651,945
China	27,116,250
East Timor	125,760
Georgia	18,361
Hong Kong	329
India	114,151,770
Indonesia	894,278
Iran	199,000
Iraq	300,000
Jordan	95
Kazakhstan	10,414
Laos	1,200,040
Malaysia	117,707
Myanmar	3,790,031
Nepal	5,277,819
Pakistan	38,848,000
Philippines	2,882,655
Sri Lanka	308,790
Syria	8,000
Taiwan	2,057
Tajikistan	15,351
Thailand	1,258,272
Turkey	161,439
Vietnam	2,425,105
Europe (%)	**460,795 (0.22%)**
Albania	95
Bulgaria	12,809
Germany	9,613
Greece	9,239
Hungary	1,000
Italy	402,796
Kosovo	400
North Macedonia	643
Poland	69
Romania	14,000
Russia	5,311
Serbia	1,000
Switzerland	1,200
United Kingdom	2,500
Ukraine	120
Oceania (%)	**137,767 (0.07%)**
Australia	133,000
Guam	94
Micronesia	173
New Zealand	1,000
Papua New Guinea	3,500

(Continued)

TABLE 2 | Continued

*† The majority of data was obtained at FAOSTAT (51). Additional sources: (7, 27, 33, 47, 53–77). Detailed methodology is presented in the **Supplementary Table 1**.*

data from buffalo herds worldwide. **Supplementary Table 1** provides detailed information regarding the source of the buffalo head count presented.

Notably, the absence of official data regarding buffalo herds in most countries mandates the retrieval of estimates from buffalo studies. Even developed countries (high-income economies), such as Canada or New Zealand, do not have any data available regarding their buffalo populations, according to their respective ministries of agriculture. Despite the absence of official information, it was possible to retrieve data on the global distribution and head count of buffaloes with some degree of confidence, mainly owing to our contacts with breeders' associations and with fellow scientists around the globe. Our data may be unreliable for two major countries, Australia and Venezuela. Australia, in particular, has two distinct herds, one feral water buffalo herd and one domesticated herd, which have

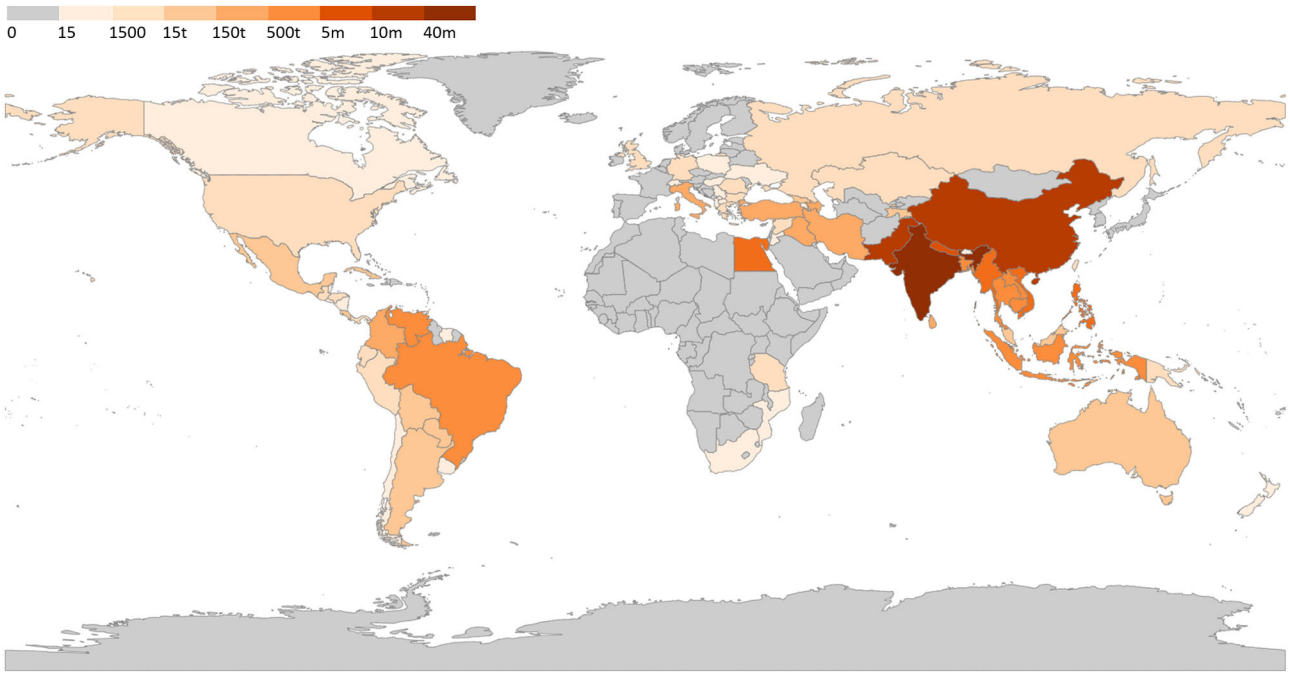

FIGURE 4 | Water buffalo population worldwide. Legend indicate number of head in each country; t, thousand; m, million. Data source available at **Supplementary Table 1**.

been increasing in numbers, owing to the use of animals for dairy purposes and the increase in animal imports in recent years (78). Recent reports estimate the population in the Northern Territory of Australia to be ∼70,000 animals; however, the herd sizes of Western Australia and Queensland are not known (79). The number of feral buffaloes in Australia was recently estimated at 120,000 animals by Lemcke (2019) *apud* Zhang (27). The Australian Buffalo Industry Council estimates that there are ∼15,000–20,000 domestic water buffaloes in the country; however, a recent report projected 13,000 farmed buffaloes (27). Therefore, the best estimate is for 133,000 buffalo head in Australia (27).

Venezuela has an increasing buffalo herd that is mostly raised in the wetland of the Llanos region, representing the second largest herd in the Americas, after Brazil (80). The herd evolved from 180,000 (81) to 350,000 animals (33). A recent report of the Venezuelan Buffalo Breeders Association reported the existence of 500,000 animals belonging to more than 190 farmers, with unverified reports suggesting that there are more than 1.8 million buffalo head in Venezuela (53). Considering the absence of official data, we considered the data reported in the aforementioned recent report. Considering the current economic situation in Venezuela, it is uncertain if this population still stands. Based on our aggregate data, we created a world map with the estimated distribution of water buffalo herds (**Figure 4**).

Buffalo herds have been subjected to major changes in recent history. **Supplementary Video 1** presents the evolution of buffalo herds in different countries from 1962 to 2017 using FAO data (51). Regarding the buffalo population trends, **Figure 5A** presents the water buffalo herd evolution in the past 50 years using data from 1968 to 2018. During these 50 years, buffalo herds worldwide almost doubled, with a population increase of 97.9%. Compared with cattle, buffalo herds had a superior trend of increase (considering the percentages), as cattle herds increased by ∼40% from 1968 to 2018. Although the buffalo herd had a greater proportional increase, the cattle population is far more numerous. The cattle herd in 1968 was around 1.06 billion reaching 1.49 billion in 2018, with an increase in the past 50 years of 425 million head (51). An evaluation of each 10-year period from 1968 to 2018 showed that the global buffalo count increased steadily at a mean rate of 14.7 ± 5.0% per 10 years. The 10-year increase was ∼11% for the periods that ended in 1978, 1998, and 2018, with sharper increases in 1988 (23%) and 2008 (15.6%). An evaluation of the buffalo population trends from the past 50 years, depending on the continent (Africa, the Americas, Asia, and Europe) is shown in **Figure 5B**. It can be noted that the buffalo population of Asia has a constantly increasing trend, while the buffalo population of Europe decreased until 1998, at which point it started to increase, but without reaching the original herd count of 1968. This phenomenon could be attributed to the fact that in Europe, only Italy constantly increased its buffalo population because of its dairy breed, cheese industry, and a market with a strong economy; on the other hand, in Eastern European countries, where buffaloes were used as draft animals for land work or carriages and had low milk yield, they were replaced

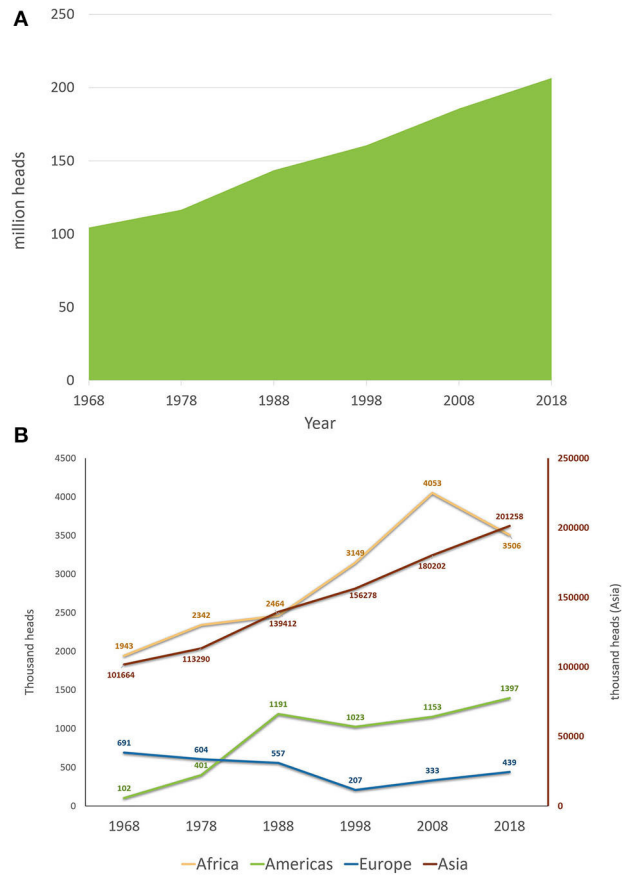

FIGURE 5 | Worldwide buffalo population trends: **(A)** worldwide buffalo population from the past 50 years (1968–2018); **(B)** worldwide buffalo population from the past 50 years (1968–2018) separated by continent. The right red axis indicates the count for Asia. America, Africa, and Europe are represented in the left axis. Source FAOSTAT (51).

by Friesian cows and mechanization. Similarly, in Asia, India, and Pakistan, the dairy breed populations increased rapidly, while in southeastern countries, the swamp buffalo populations decreased. Countries in the Americas and Africa experienced increasing trends; however, both areas experienced a 10-year period when their buffalo populations decreased (1988–1998 in the Americas and 2008–2018 in Africa).

BUFFALO MILK PRODUCTION

In many countries, buffalo breeding has a family character in which the herds are represented by a few animals used for subsistence. In many societies, buffalo milk is acquiring more and more visibility linked to its intrinsic value as a dairy product, as it can not only be consumed directly, as is the case in the Asian continent, but can also be transformed into dairy products of high commercial demand (36, 40). According to FAO data, the buffalo milk production accounted for ∼15.14% of the global fresh milk production in 2018, while cattle milk accounted for more than 80% (**Table 3**). **Supplementary Video 3** presents the buffalo milk production (in tons of milk per year) in different countries from 1962 to 2017 using FAO data (51).

The comparison of these percentages within each continent produces interesting scenarios. For example, in Asia where buffalo milk accounts for 35.30% of the total milk production, buffaloes are the major milk-producing animal in India and Pakistan; additionally, there are higher populations of dairy buffaloes than dairy cows in Egypt and Nepal (51). The top 10 largest producers of buffalo milk worldwide (India, Pakistan, China, Egypt, Nepal, Italy, Myanmar, Iran, Colombia, and Brazil) are responsible for 97.59% of the global production (**Table 4**). It should be considered that, given the lack of production data for over 50 of the 77 countries in which buffaloes are bred, these values represent an estimate of the real production potential of the species; in fact, of 208 million buffaloes, 7.48 million are

TABLE 3 | Buffalo, camel, cow, goat, and sheep milk whole fresh produced in 2018 for each region.

Milk, whole fresh†		Buffalo	Camel	Cow	Goat	Sheep	Total
World	Tons	127,658,734	3,137,071	683,217,056	18,712,088	10,631,058	843,356,007
	%	15.14%	0.37%	81.01%	2.22%	1.26%	100.00%
Asia	Tons	124,958,493	262,233	213,201,098	10,627,509	4,924,398	353,973,731
	%	35.30%	0.07%	60.23%	3.00%	1.39%	100.00%
America	Tons	189,739	NA	184,304,156	779,806	90,871	185,364,572
	%	0.10%	NA	99.43%	0.42%	0.05%	100.00%
Europe	Tons	390,137	76	220,377,066	2,722,332	3,168,166	226,657,777
	%	0.17%	0.00%	97.23%	1.20%	1.40%	100.00%
Oceania	Tons	NA	NA	30,706,258	39	NA	30,706,297
	%	–	–	100.00%	0.00%	–	100.00%
Africa	Tons	2,120,365	2,874,762	34,628,478	4,582,402	2,447,623	46,653,630
		4.54%	6.16%	74.22%	9.82%	5.25%	100.00%

† *Data from 2018 (51). The percentage (%) of animal species milk was calculated according to each geographical area. NA, not available.*

bred in countries in which there are no reported data for buffalo milk production.

To date, only Italy has established an official system for the traceability of milk production and the entire buffalo supply chain since 2014 (83), which provides real-time information on milk production. The establishment of this system was favored by the economic value that the supply chain represents for the country. In fact, Italy, which ranks fifth globally in terms of its milk production, produces 97% of the buffalo milk produced in Europe (**Table 4**); only the buffalo mozzarella that is protected under the EU's Protected Designation of Origin (PDO) scheme, is estimated at a sales value of 766 million euros (84).

According to FAO data, the global buffalo milk production increased by 32.57% from 2011 to 2018 (from 96 to 127 million tons); on the contrary, cattle milk increased by 10.67% (from 617 to 683 million tons). Many factors that characterize the various breeding activities worldwide make it very complex to estimate the actual production of fresh buffalo milk. The breed, production purpose (milk, meat, work), breeding type (extensive and intensive), feeding techniques, seasonality at different latitudes, age at first birth, weaning techniques, reproductive techniques applied, lactation duration, milking of the subjects, genetic selection applied, and the longevity owing to economic sustainability linked to the commercial value of the milk are some of these factors (42, 85).

There are many factors that affect the milk yield in different species; breed, genetic background, season and period of calving, health status, and environmental factors such as feeding, climate conditions, and welfare are some of these factors. These factors are also important in buffalo species; however, the genetic potential is the most important factor, as exemplified in the Italian Mediterranean, Murrah, and Nili-Ravi breeds. A lot of work has underwent in selecting the most appropriate female lines by recording animals and creating genetic centers with selected bulls for the spread of artificial insemination. Milk production during lactation is a continuous physiological function. In buffalo species, the lactation curve, which is a graphical representation of milk yield during lactation, shows a rapid increase, peaks, reaches a plateau phase, and declines gradually until the end of lactation (86). In economic terms, the most important phase is the persistence of lactation, which means the ability to maintain milk production at high levels after the peak of lactation (87). Genetic and environmental influences on the persistence of lactation were studied in Indian Murrah buffaloes by Geetha et al. (88) and in Nili-Ravi buffaloes by Chaudhry et al. (89), who showed the effect of parity and of lactation length on persistency. The effects of parity, age, and calving season were also studied in the Bulgarian (90) and the Italian Mediterranean breeds (91, 92).

Compared to cow's milk, buffalo milk is approximately twice as high in fat content and ∼30% higher in total solids; therefore, if the calculation was made on a dry matter basis, the importance of buffalo milk worldwide would be even greater (93). In terms of energy, 1 kg of buffalo milk equals 5.10 Mj, which is much higher than the 2.90 Mj/kg of 1 kg of cow's milk (94). The water buffalo is of the most productive domestic animals, has a longer productive life when compared to cattle, and is economically important, especially for small-scale producers in developing countries. Given its characteristics of resilience and adaptability to tropical climates (95), the buffalo represents an important food source of animal origin.

TABLE 4 | Current data on buffalo milk production worldwide.

Region/country	Milk production[α]	%[β]	References
Worldwide	**127,658,734**	**100.00%**	
Africa	**2,120,365**	**1.66%**	†
Egypt	2,120,365	100.00%	†
Americas	**189,739**	**0.15%**	
Brazil	87,472	46.10%	Data estimated (82)
Colombia	102,267	53.90%	Data estimated (76)
Asia	**124,958,493**	**97.88%**	
Bangladesh	35,691	0.03%	†
Bhutan	282	0.00%	†
Brunei	184	0.00%	†
China	3,003,323	2.40%	†
Georgia	6,186	0.00%	†
India	91,817,140	73.48%	Official data[γ]
Indonesia	71,166	0.06%	†
Iran	129,904	0.10%	†
Iraq	49,893	0.04%	†
Malaysia	8,190	0.01%	†
Myanmar	193,841	0.16%	†
Nepal	1,338,277	1.07%	Official data[γ]
Pakistan	28,109,000	22.49%	Official data[γ]
Sri Lanka	85,914	0.07%	Official data[γ]
Syrian	6,300	0.01%	Official data[γ]
Turkey	75,742	0.06%	Official data[γ]
Viet Nam	27,460	0.02%	†
Europe	**390,137**	**0.31%**	
Albania	12	0.00%	†
Bulgaria	11,753	3.01%	Official data[γ]
Greece	402	0.10%	†
Italy	377,970	96.88%	Official data (65)

[α] *Water buffalo milk production worldwide in tons (2018 data).* [β] *Percentage is related to the contribution of each continent to the global buffalo milk production and from each country to their continent total milk production.* [†] *FAO data based on imputation methodology (51).* [γ] *Official data retrieved from Faostat 2019 (51).*

Buffalo milk production in several countries is driven for processing and for the cheese industry. The mozzarella cheese is recognized and largely consumed worldwide as an adulteration of the original *Mozzarella* cheese, which is made exclusively from buffalo milk. Only in 1993, was mozzarella from buffalo recognized as "*Mozzarella di Bufala Campana* PDO." The PDO certification pertains to buffaloes bred in the Campania and Lazio regions of Italy that produce the milk used for the production of the famous mozzarella cheese; what this essentially means is that this cheese has to be produced in the defined areas only from fresh buffalo milk from the Italian Mediterranean breed, registered in the Buffalo Genealogical Book (36). The mozzarella industry in Italy resulted from 34,990 recorded females of the Italian Mediterranean breed, which account for ∼30% of the total dairy buffalo population (this percentage does not exist in any

other country) and have a mean production of 2,356 kg milk in 270 days of lactation, with 8% fat and 4.63% protein (96).

CONCLUSION

There is a surprising lack of data regarding buffalo herds worldwide. In this study, we reviewed the literature and established a more appropriate buffalo distribution and population count. Our results showed that buffaloes are present in 77 countries in five continents with a population of more than 208 million head. Considering buffalo characteristics such as rusticity and productivity, we believe that buffalo production should be expanded worldwide, especially in developing countries with adequate natural conditions. This amazing and subutilized animal can become more productive, and should be promoted as a target species to be used in smallholder production systems. Governments worldwide must look carefully to this amazing animal and start developing projects to introduce this species in regions with favorable conditions, in order to substitute cattle herds.

AUTHOR CONTRIBUTIONS

All the authors conceived, research, write, and revised this manuscript.

FUNDING

This work was supported by Ricerca Corrente IZS ME 8/18 RC: Study of animal welfare and sustainability of the dairy buffalo production chain through a multidisciplinary approach CUP C75B18000630001. Financed by the Italian Ministry of Health.

ACKNOWLEDGMENTS

This work is dedicated to all buffalo farmers around the world, with a special honor to Victor Makol Julien "o reizinho do búfalo" (*in memorian*). Those passionate and dedicated workers are the main responsible for the buffalo expansion and production. AM is grateful to CNPq for his research productivity fellowship.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.570413/full#supplementary-material

Supplementary Table 1 | Sources and methodology for the aggregate data of the world population of water buffalo (*Bubalus bubalis*) per country.

Supplementary Video 1 | This video presents the buffalo herd evolution (in number of animals) in the different countries from 1962 to 2017 using the data from FAO (51). Video produced through FlourishTM platform (Kiln Enterprises Ltd, London, UK). Available online at: https://flourish.studio/.

Supplementary Video 2 | Herd of docile and curious mixed-breed buffaloes in Santarém, Amazon, Brazil.

Supplementary Video 3 | This video presents the buffalo milk production (in tons of milk per year) in the different countries from 1962 to 2017 using the data from FAO (51). Video produced through FlourishTM platform (Kiln Enterprises Ltd, London, UK). Available online at: https://flourish.studio/.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 02 December 2020
doi: 10.3389/fvets.2020.577775

"Which Factors Affect Pregnancy Until Calving and Pregnancy Loss in Buffalo Recipients of *in vitro* Produced Embryos?"

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OPEN ACCESS

Edited by:
Domenico Vecchio,
Istituto Zooprofilattico Sperimentale del Mezzogiorno, Italy

Reviewed by:
Bouchra El Amiri,
Institut National de la Recherche Agronomique de Settat, Morocco
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Specialty section:
This article was submitted to Animal Reproduction - Theriogenology, a section of the journal Frontiers in Veterinary Science

Received: 29 June 2020
Accepted: 10 October 2020
Published: 02 December 2020

Citation:
Saliba WP, Gimenes LU, Drumond RM, Bayão HXS, Di Palo R, Gasparrini B, Rubessa M, Baruselli PS, Sales JNS, Bastianetto E, Leite RC and Alvim MTT (2020) "Which Factors Affect Pregnancy Until Calving and Pregnancy Loss in Buffalo Recipients of in vitro Produced Embryos?" Front. Vet. Sci. 7:577775. doi: 10.3389/fvets.2020.577775

In vitro embryo production and embryo transfer (ET) in buffaloes has been developed for decades. However, most studies are focused on the donor or laboratory improvements, and there is a lack of reports regarding the recipients. Therefore, our aim was to investigate factors associated to pregnancy (P/ET), pregnancy loss (PL), and calving rates in buffalo recipients. The studied factors were season, recipient parity, the synchronization protocol, the CL diameter, asynchrony between the embryo and the recipient, the day of the recipient estrous cycle, the embryo (fresh vs. vitrified), the day of embryo development, and the embryo stage. These retrospective data, from a program of *in vitro* produced embryos, were analyzed by logistic regression, and the odds ratio was also estimated. Two factors were related to P/ET and the calving rate: (1) progesterone associated to estradiol plus eCG protocol for fixed time ET tended to affect positively P/ET on day 30 (41.9 vs. 36.1%, respectively; $P = 0.07$; AOR = 1.28) and P/ET on day 60 (37.8 vs. 36.1%, respectively; $P = 0.09$; AOR = 1.08) compared to the Ovsynch protocol; and (2) the CL diameter (≥14.5 mm) at transfer increased P/ET on day 30 (47.4 vs. 32.5%; $P < 0.01$; AOR = 1.87) and on day 60 (45.3 vs. 27.7%; $P < 0.01$; AOR = 2.16), and also the calving rate (37.9 vs. 21.7%; $P < 0.01$; AOR = 2.20). PL was greater when ET was done in the nonbreeding season compared to the breeding season (PL 30–60: 12.8 vs. 0.0%, $P = 0.01$; AOR > 999.99; PL 60-calving: 26.8 vs. 3.6%, $P = 0.03$; AOR = 9.90; and PL 30-calving: 36.2 vs. 3.6%, $P = 0.01$; AOR = 15.30). In conclusion, the data of our study indicated that the synchronization protocol, the CL diameter, and ET during the breeding season impacted the reproductive efficiency of buffalo recipients.

Keywords: embryo transfer, OPU, IVEP, fresh embryos, vitrification, embryo/fetal mortality

INTRODUCTION

Brazil is home to 0.67% of the world buffalo herd, with a population of about 1,390,066 (1). Buffaloes have advantages due to their rusticity, adaptability, and the quality of their main products, the milk, and the meat.

Several reproductive biotechnologies, such as superovulation for *in vivo* embryo production (SOV) or ovum pick-up (OPU) for *in vitro* embryo production (IVEP), have been used in order to obtain animals with better performance and high genetic merit (2). Unfortunately, the efficiency of superovulation for *in vivo* embryo transfer (ET) in buffaloes is low when compared to cattle (3). For this reason, the association of OPU and IVEP is currently the most competitive and suitable solution for this purpose, reducing the interval between generations (3, 4).

The strategy of embryo transfer (ET) has enabled genetic progress through maternal lineage and also contributed to the control of infectious diseases (5). Besides the use of fresh embryos, both conventional freezing and vitrification were used for cryopreservation, resulting in pregnancy and calving (6–12), although low pregnancy/embryo transfer [P/ET; 10.5–26.9% (8, 10, 11)] and calving rates [10.0–25% (8, 9, 11, 12)] were generally reported.

In cattle, the recipient plays a critical role in the success of ET programs (13, 14). Several factors are associated with P/ET and calving rates, such as the diameter of corpus luteum (CL), synchronization protocols, season (14), the synchrony between the embryo stage and the recipient, embryo quality, and recipient management (15). However, in buffaloes, only one retrospective study regarding recipients was carried out (16). In that article, asynchrony >12 h and the use of grade I embryos were the factors that enhanced P/ET; the CL diameter also tended to increase P/ET. Since then, few studies were focused on buffalo recipients and, to our knowledge, none were conducted to evaluate multiple factors influencing pregnancy and calving outcomes or pregnancy loss after ET using *in vitro* produced embryos.

Therefore, the aim of the present study was a retrospective analysis of a dataset obtained from an ET program in order to determine which factors can affect pregnancy, calving rates, and pregnancy loss in buffalo recipients.

MATERIALS AND METHODS

Experimental Location and Animals

The dataset was obtained from 201 ovum pick-up (OPU) sessions and 184 ETs carried out at Cenatte Embriões LTDA, located in the city of Pedro Leopoldo, Minas Gerais, Brazil (latitude: 19°37′12″ south, longitude: 44°2′38″ west).

A total of 73 crossbred Murrah buffalo females (23 nulliparous and 50 multiparous), healthy, nonpregnant, and cycling were used as oocyte donors and embryo recipients. The animals were kept under grazing of *Brachiaria decumbens* and *B. brizantha* with free access to water and mineral salt. During the autumn and winter periods, all animals were supplemented with elephant grass silage to meet the demand of each category (17).

Management of Oocyte Donors

All females were used as oocyte donors along the experimental period, and groups of 6–36 animals were submitted to 11 replicates with a minimum interval of 19 days, for 8 months, without any hormonal stimulation.

The animals were contained and submitted to epidural anesthesia, using 3 ml of 2% lidocaine (Lidovet®, Bravet). OPU procedure was performed using transvaginal ultrasound equipment (Aloka 500®, Tokyo, Japan; frequency 5 MHz) coupled to a system with an 18G disposable hypodermic needle (BD) and a 1.7-mm-diameter and 80-cm-long Teflon aspiration line (WTA, Watanabe Applied Technology). The vacuum pressure used was 60–65 mmHg (10 and 12 ml of water/minute). A warm solution (37°C) containing PBS with sodium heparin (5,000 UI/L; Parinex®, Hypolabor), 1% fetal bovine serum (FBS, Nutricell), and 0.1% antibiotic solution (amikacin) was used to collect the aspirated oocytes from follicles larger than 2 mm.

After OPU, cumulus-oocyte complexes (COCs) were searched and classified under a stereomicroscope. TCM 199 with 5% FBS, pyruvate (0.2 mM), and amikacin (83.4 mg/ mL) was used to wash the COCs collected. The criteria used for the evaluation of COC were the presence, the number of layers, and the degree of compaction of *Cumulus* cells, as well as the appearance of the cytoplasm in color, homogeneity, and integrity. Up to their parameters, the COCs were classified as follows: grade I (three or more layers of cells of the compact cumulus and homogeneous cytoplasm), II (one to three layers of cumulus cells covering totally or partially the oocyte and with homogeneous or heterogeneous cytoplasm), and III (one layer of cumulus cells covering the oocyte totally or partially and with cytoplasm homogeneous or heterogeneous), denuded, degenerated, expanded, and atretic (18). For *in vitro* embryo production (IVEP), only oocytes grades I, II, and III (viable oocytes) were used.

Viable oocytes were maintained in 1.2 ml cryovials (Corning) containing an oocyte maturation medium, inside an incubator for embryo transport (WTA) at 38.5°C with gas mixture (5.5% CO_2, 5.0% O_2, and 89.5% N_2), until the arrival to the laboratory. The maximum transport time to the laboratory was 6 h after the beginning of OPU.

In vitro Embryo Production (IVEP)

In the laboratory, groups of 10 to 12 oocytes were transferred to 50-μL drops of a maturation medium, composed of TCM199 with sodium bicarbonate supplemented with 10% FBS, pyruvate (0.2 mM), amikacin (83.4 mg/ mL), 0.5 μg of FSH (Folltropin), 5 μg of LH (Lutropin), 1 μg of 17 α-estradiol, and supplemented with 50 μM of 2-mercaptoethanol, covered with sterile mineral oil. Oocytes were incubated for 20–24 h, at 38.7°C, with 5.5% CO_2 in air and saturated humidity.

One hour before the end of the maturation period, the semen from a tested bull was thawed at 35°C for 20 s and deposited on a Percoll gradient (30, 60, and 90%) for sperm selection. Centrifugation was performed at 9,000 × g for 2 min, and, subsequently, the pellet was washed twice in a total alkaline phosphatase medium (TALP), at 4,500 × g for 1 min. The

inseminating dose was adjusted to 1.2 million sperm/mL. After IVM, the oocytes were washed in a TCM199 HEPES medium containing 0.3% BSA, pyruvate (0.2 mM), and amikacin (83.4 mg/mL). Then they were washed in a TALP fertilization medium with 0.5% BSA, pyruvate (0.2 mM), amikacin (83.4 mg/ mL), heparin (20 μg/ mL), and PHE solution (2 μM of penicillamine, 1 μM of hypotaurine, and 0.25 μM of epinephrine), and groups of 10–12 oocytes were co-cultured with sperm in 50 μL drops of this same medium, under the same conditions of atmosphere, humidity, and temperature used for IVM for 18–20 h.

After fertilization (D0), the presumptive zygotes were denuded, and groups of 10–12 were transferred to 100 μL drops containing a SOFaa medium with 3% FBS under mineral oil, during 5–7 days at 38.7°C, with 5.5% CO_2, 5.0% O_2, and 89.5% N_2 in saturated humidity. Forty-eight hours after fertilization, the embryo cleavage was evaluated. There was no change of medium throughout the *in vitro* culture. On 5th to 7th days after fertilization (D5–D7, respectively), the blastocyst rate (blastocysts/viable oocytes) was calculated.

Embryo Evaluation and Preparing for Fresh or Vitrified Transfer

Embryos were morphologically evaluated and classified according to the IETS manual (19), in terms of developmental stage: early blastocyst, blastocyst, expanded blastocyst, and hatched blastocyst; and quality: grade I (excellent or good), II (fair), III (poor), or IV (degenerated).

Only grade I D5 and D6 embryos, varying from early to hatched blastocysts, were transferred. The embryos were loaded in 0.25-mL straws containing a maturation washing medium (TCM 199 HEPES, 5% FBS, pyruvate, and amikacin) and were maintained in the portable incubator until transfer to recipients.

Vitrification was performed when the number of embryos exceeded the number of recipients. Only grade I D5, D6, and D7 embryos, varying from expanded to hatched blastocysts, were vitrified on Cryotop® according to the method described by De Rosa et al. (20). Briefly, the embryos were placed in a solution containing 7.5% ethylene glycol and 7.5% dimethyl sulfoxide (DMSO), prepared in TCM 199 HEPES supplemented with 20% FBS, for 3 min and then were transferred to a second solution containing 16.5% ethylene glycol, 16.5% DMSO prepared in FBS with 0.5 M sucrose. After 25 s, the embryos were kept in a volume of <0.1 μL of vitrification solution and loaded on Cryotop®, which was immediately immersed in liquid nitrogen. The warming procedure was carried out by immersing the top of Cryotop® containing the embryos in a 0.25 M sucrose solution. After 1 min, the embryos were transferred to a 0.15 M sucrose solution for 5 min and then loaded in 0.25-mL straws for transfer to recipients.

Recipients Management

All females previously used as oocyte donors were also used as recipients, along 24 months, totalizing 184 procedures divided in 18 replicates (fresh: $n = 10$; vitrified: $n = 8$ replicates).

All recipients were submitted to one of the two following synchronization protocols: Ovsynch (21) or estradiol plus progesterone based (22). Briefly, the Ovsynch protocol consisted of 0.5 mg of lecirelin (Gestran Plus®, Tecnopec) on a random day of estrous cycle. Seven days later, 0.5 mg of cloprostenol (Sincrosin®, MSD Animal Health) was administered. A second dose of lecirelin (0.25 mg) was injected 48 h later. The second protocol consisted in the insertion of an intravaginal progesterone device, used for 8 days (multiparous) or 16 days (nulliparous) associated to 2 mg of estradiol benzoate (Estrogin®, Biofarm) and 530 mg of cloprostenol, on a random day of estrous cycle. Nine days later, the devices were removed and 400 IU of eCG (Folligon®, MSD Animal Health) and PGF2α were administered to all recipients. Finally, after 48 h, the recipients were treated with 0.5 mg of lecirelin (Gestran Plus®, Tecnopec).

Six or seven days later, ovulation was evaluated by the presence of a CL, which was measured by ultrasound (Aloka 500®, Tokyo, Japan; frequency 5 MHz), and fresh or vitrified embryos were transferred in the uterine horn ipsilateral to CL, by a nonsurgical method. Only one embryo was transferred in each recipient. Asynchrony between embryos and recipients was ±1 day.

Monitoring of Pregnancy and Birth

Pregnancies were monitored by ultrasound (Aloka 500, Tokyo, Japan; frequency 5 MHz) 30 and 60 days after the expected ovulation. At the due day, the animals were evaluated for labor stages, dystocia, and placental retention. The newborns were weighed, the sex determined, and suckling reflex and umbilicus were examined.

P/ET was calculated as the percentage of pregnant females, diagnosed on days 30 (P/ET 30) and 60 (P/ET 60), on the total number of the recipients. The calving rate was calculated as the percentage of females calving out on the total number of recipients. PL was calculated as the percentage of nonpregnant females, from days 30 to 60, day 60 to calving, and day 30 to calving, in relation to females pregnant on days 30, 60, and 30, respectively.

Statistical Analysis

Data concerning *in vitro* embryo production were shown according to descriptive statistics, except by analysis of birth weight associated to sex of calves, which was analyzed by logistic regression, by using the GLIMMIX procedure in SAS for Windows (23). Data regarding recipients were analyzed by univariate and multivariate logistic regression, using the LOGISTIC procedure (23). All groups with fewer than 10 recipients were excluded from the analysis. For pregnancy and calving rate analysis, the following were excluded: asynchrony (−1 day, $n = 7$), day of embryo development (day 7, $n = 7$), and embryo stage (hatched blastocyst, $n = 9$). For pregnancy loss, the following were excluded: asynchrony (−1 day, $n = 2$), day of embryo development (day 7, $n = 2$), and embryo stage (early blastocyst, $n = 5$; blastocyst, $n = 8$; hatched blastocyst, $n = 5$). Adjusted odds ratio (AOR) and 95% confidence interval (CI) were generated by logistic regression. Odds ratio is a measure of association between a treatment and an outcome and represents the odds that an outcome will occur given a particular treatment compared to the odds of the outcome occurring in the absence of that treatment or another treatment (24). Results are presented as

percentages and AOR. $P \leq 0.05$ were considered significant, and those $0.05 \leq P \leq 0.10$ were considered as tendency.

RESULTS

Overall Embryo Rate, P/ET, Calving Rates, and PL

A total of 998 oocytes were recovered from 201 OPU sessions (5.0 ± 0.5/donor), of which 584 were viable oocytes (2.9 ± 0.3/donor). A total of 318 structures cleaved (1.6 ± 0.2/donor), resulting in a cleavage rate of 54.5%. The blastocyst rate was 44.9% producing 262 embryos (1.3 ± 0.1/donor), of which 229 were grade I, 5 grade II, and 28 grade III.

From the total embryos produced, 114 were transferred fresh resulting in P/ET 30, P/ET 60, and a calving rate of 43.0% (49/114), 41.2% (47/114), and 35.1% (40/114), respectively. Pregnancy loss between days 30 and 60 was 4.1% (2/49), between day 60 and calving was 14.9% (7/47), and between day 30 and calving was 18.4% (9/49). The mean gestation length was 310.2 ± 0.5 days. All calvings were eutocic, and the average weight of calves born was 32.6 ± 0.2 kg, being lower for females (31.4 ± 0.4 kg; $n = 20$) than for males (33.8 ± 0.6 kg; $n = 20$; $P < 0.01$). Concerning postpartum occurrences, two females showed retained placenta (5%), two cervix prolapse (5%), and one rejected her calf (2.5%).

For vitrified embryos, 70 were transferred and P/ET 30, P/ET 60, and the calving rate were, respectively, 37.1% (26/70), 31.4% (22/70), and 24.3% (17/70). Pregnancy loss between days 30 and 60 was 15.4% (4/26), between day 60 and calving was 22.7% (5/22), and between day 30 and calving was 34.6% (9/26). The mean gestation length was 314.5 ± 0.9 days, and one of the animals presented dystocia. The average weight of calves born was 33.8 ± 0.2 kg, being similar in females (33.6 ± 0.7 kg; $n = 10$) and males (34.0 ± 0.4 kg; $n = 7$; $P = 0.33$). In the postpartum period, retained placenta was not observed in any recipient, and one of the calves had suckling difficulty in the first 24 h (5.9 %).

Factors Associated to Pregnancy and Calving Rate in Buffalo Recipients

Pregnancy and calving rates were significantly associated to the synchronization protocol and the CL diameter. Reproductive season, parity, asynchrony, day of recipient estrus, type of embryo, and day or stage of embryo development did not affect P/ET 30, P/ET 60, or the calving rate (**Table 1**).

When progesterone associated to estradiol plus the eCG-based protocol was compared to Ovsynch, there was a tendency of greater P/ET 30 (41.9 vs. 36.1%; $P = 0.07$; AOR = 1.28) and P/ET 60 (37.8 vs. 36.1%; $P = 0.09$; AOR = 1.08).

Recipients with a CL diameter larger than or equal to 14.5 mm had increased P/ET 30 (47.4 vs. 32.5%; $P < 0.01$; AOR = 1.87), P/ET 60 (45.3 vs. 27.7%; $P < 0.01$; AOR = 2.16), and a calving rate (37.9 vs. 21.7%; $P < 0.01$; AOR = 2.20), when compared to those with CL < 14.5 mm.

Factors Associated to Pregnancy Loss in Buffalo Recipients

Pregnancy loss (PL) was associated only to the season of the embryo transfer. No other factor influenced PL from days 30 to 60 (PL 30–60), day 60 to calving (PL 60-C), or day 30 to calving (PL 30-C; **Table 2**).

PL was greater when ET was performed in the nonbreeding season compared to the breeding season (PL 30–60: 12.8 vs. 0.0%, $P = 0.01$; AOR > 999.99; PL 60-C: 26.8 vs. 3.6%, $P = 0.03$; AOR = 9.90; and PL 30-C: 36.2 vs. 3.6%, $P = 0.01$; AOR = 15.30).

DISCUSSION

The interest in large-scale production of buffalo embryos has been growing worldwide (3). Despite this, embryo recipients are still neglected compared to donors of oocytes and embryos, and few studies were conducted to clarify aspects related to pregnancy and even fewer to clarify aspects related to calving and pregnancy loss. To our knowledge, the present retrospective nonrandomized study is the first to achieve this purpose for buffalo recipients.

In this study, the CL diameter was the main factor that influenced pregnancy until calving. Although controversial, several studies in cattle demonstrated that P/ET was higher when recipients had larger CL, mainly due to an increase in progesterone levels (14). High levels of progesterone allow embryo elongation and increase interferon τ production by the conceptus, avoiding luteolysis in the critical period of maternal recognition of pregnancy (25). In buffaloes, few trials were conducted to evaluate these features. In a study with synchronization of ovulation protocol for fixed time artificial insemination (AI) in buffaloes, a significant and positive correlation between the diameter of dominant follicle at AI and the CL diameter 10 days later, ovulation rate, and pregnancy/AI was found, besides a negative correlation with embryo mortality (22). In another trial (26), cows categorized with a larger diameter of pre-ovulatory follicles on the day of estrus had a positive correlation with the CL diameter and progesterone concentration, and these variables were greater in pregnant than in nonpregnant females. Interestingly, in that work, nonpregnant females had CL diameters close to 15 mm (and progesterone < 1 ng/ mL), corroborating Di Palo et al. [1990, revised in (27)] who reported higher fertility in buffaloes with CL ≥ 15 mm. Therefore, the data of the present study support that the CL diameter (≥14.5 mm) can be used as a parameter to select the recipients in buffaloes in order to obtain better outcomes.

Another finding associated to P/ET 30 and P/ET 60 was the synchronization protocol. The Ovsynch protocol has been used in buffaloes to synchronize ovulation for fixed time artificial insemination (21). As previously shown in buffaloes and cattle, the main disadvantage of this protocol is the response to the first GnRH, which can depend on the status of the dominant follicle in the beginning of treatment (28–30). Nevertheless, in anestrus animals, the efficiency of this protocol results in

TABLE 1 | Factors associated to pregnancy (P/ET) on days 30 and 60, and calving rate in buffalo recipients.

		P/ET				Calving rate	
	N	30 days	OR (95% CI)*	60 days	OR (95% CI)	Calving	OR (95% CI)
Season							
Breeding season	80	35.0	Ref.#	35.0	Ref.	33.8	Ref.
Nonbreeding season	104	45.2	1.53 (0.84–2.79)	39.4	1.21 (0.66–2.21)	28.9	0.80 (0.43–1.49)
Recipient parity							
Cow	134	40.3	Ref.	36.6	Ref.	28.4	Ref.
Heifer	49	40.8	1.02 (0.56–1.99)	38.8	1.10 (0.56–2.16)	36.7	1.47 (0.74–2.93)
Protocol							
Ovsynch	36	**36.1**[b]	Ref.	**36.1**[b]	Ref.	33.3	Ref.
P4 + EB[$]	148	**41.9**[a]	1.28 (0.6–2.71)	**37.8**[a]	1.08 (0.51–2.30)	30.4	0.87 (0.40–1.90)
CL diameter							
<14.5 mm	83	**32.5**[B]	Ref.	**27.7**[B]	Ref.	**21.7**[B]	Ref.
≥14.5 mm	95	**47.4**[A]	1.87 (1.01–3.44)	**45.3**[A]	2.16 (1.15–4.04)	**37.9**[A]	2.20 (1.13–4.29)
Asynchrony							
0	47	34.0	Ref.	29.8	Ref.	21.3	Ref.
+1	122	42.6	1.44 (0.71–2.90)	40.2	1.58 (0.77–3.26)	34.4	1.94 (0.88–4.29)
Day of recipient estrous cycle							
7	73	41.1	Ref.	38.4	Ref.	31.5	Ref.
6	111	40.5	0.98 (0.54–1.78)	36.9	0.94 (0.51–1.73)	30.6	0.96 (0.51–1.82)
Embryo							
Vitrified	70	37.1	Ref.	31.4	Ref.	24.3	Ref.
Fresh	114	43.0	1.28 (0.69–2.35)	41.2	1.53 (0.82–2.87)	35.1	1.69 (0.86–3.29)
Day of embryo development							
6	114	36.5	Ref.	33.1	Ref.	26.1	Ref.
5	55	48.2	1.61 (0.84–3.11)	46.3	1.75 (0.90–3.38)	40.7	1.95 (0.98–3.86)
Embryo stage							
Expanded blastocyst	135	40.7	Ref.	37.8	Ref.	30.4	Ref.
Blastocyst	18	44.4	1.16 (0.43–3.14)	38.9	1.05 (0.38–2.88)	38.9	1.46 (0.53–4.03)
Early blastocyst	15	33.3	0.73 (0.24–2.25)	33.3	0.82 (0.27–2.55)	26.7	0.83 (0.25–2.77)

**OR, odds ratio; CI, confidence interval.*
#Ref., reference point odds ratio = 1.0.
$P4 + EB: progesterone associated to estradiol plus eCG protocol.
A ≠ B (P ≤ 0.05); a ≠ b (0.05 ≤ P ≤ 0.10).

low follicular response and conception rates (21). To overcome this negative effect, treatments based on the association of progesterone and estradiol esters, like in cattle, have been also applied to buffalo reproductive management, with positive pregnancy results independent of animal cyclicity (22). Besides that, in the present study, eCG was associated to P4 plus the E2-based protocol. The beneficial effect of eCG on the maximum diameter of the dominant follicle, the ovulation rate, the CL diameter, CL development, and progesterone production is well-documented in cattle and buffaloes, culminating with higher P/ET (14, 31, 32).

Concerning the PL, our data demonstrated an association between PL and the season when the embryos were transferred. When ET occurred in the nonbreeding season (from October to April), a consistent embryo and fetal mortality was observed along all periods analyzed. A seasonal effect on late embryo mortality (between 26 and 40 days of pregnancy) was previously described by Campanile et al. (33). The authors recorded 45%

TABLE 2 | Factors associated to pregnancy loss (PL) from days 30 to 60, day 60 to calving, and day 30 to calving in buffalo recipients.

		PL					
	N	Days 30–60	OR (95% CI)*	60 days–calving	OR (95% CI)	30 days–calving	OR (95% CI)
Season							
Breeding season	28	**0.0**[A]	Ref.#	**3.6**[A]	Ref.	**3.6**[A]	Ref.
Nonbreeding season	47	**12.8**[B]	>999.90 (<0.01–∞)	**26.8**[B]	9.90 (1.20–81.80)	**36.2**[B]	15.30 (1.91–122.80)
Recipient parity							
Heifer	20	5.0	Ref.	5.3	Ref.	10.0	Ref.
Cow	54	9.3	1.93 (0.21–17.70)	22.4	5.21 (0.62–43.52)	29.6	3.79 (0.79–18.27)
Protocol							
Ovsynch	13	0.0	Ref.	7.7	Ref.	7.7	Ref.
P4 + EB[$]	62	9.7	>999.90 (<0.01–∞)	19.6	2.93 (0.34–25.02)	27.4	4.53 (0.55–37.58)
CL diameter							
≥14.5 mm	45	4.4	Ref.	16.3	Ref.	20.0	Ref.
<14.5 mm	27	14.8	3.74 (0.64–21.97)	21.7	1.43 (0.39–5.14)	33.3	2.00 (0.68–5.91)
Asynchrony							
0	16	12.5	Ref.	28.6	Ref.	37.5	Ref.
+1	52	5.8	0.43 (0.07–2.83)	14.3	0.42 (0.10–1.71)	19.2	0.40 (0.12–1.35)
Day of recipient estrous cycle							
7	30	6.7	Ref.	17.9	Ref.	23.3	Ref.
6	45	8.9	1.37 (0.23–7.97)	17.1	0.95 (0.27–3.35)	24.4	1.06 (0.36–3.14)
Embryo							
Fresh	49	4.1	Ref.	14.9	Ref.	18.4	Ref.
Vitrified	26	15.4	4.27 (0.73–25.12)	22.7	1.68 (0.47–6.05)	34.6	2.35 (0.79–6.96)
Day of embryo development							
5	26	3.9	Ref.	12.0	Ref.	15.4	Ref.
6	42	9.5	2.6 (0.28–24.93)	21.1	2.0 (0.47–8.22)	28.6	2.2 (0.63–7.74)
Embryo stage							
Expanded blastocyst	57	8.8	–	19.2	–	26.3	–

*OR, odds ratio; CI, confidence interval.
[#]Ref.: reference point odds ratio = 1.0.
[$]P4 + EB: progesterone associated to estradiol plus eCG protocol.
∞ > 999.99.
A ≠ B ($P \leq 0.05$).

embryo mortality when the buffaloes were inseminated during periods of increasing daylight. These authors explained this result with a reduced CL capacity to secrete progesterone. Moreover, Vecchio et al. (34) reported high incidence of fetal mortality (between 45 and 90 days of pregnancy) in buffaloes naturally mated in increasing daylight. Overall, these works confirmed our data and indicate that new trials should be performed in order to overcome early and late PL in buffaloes when reproductive strategies are conducted during the nonbreeding season.

Although in the present study progesterone levels were not analyzed, the factors affecting P/ET, the calving rate, and PL herein found collectively suggest that strategies that favor the increase in progesterone levels (synchronization protocols based on progesterone associated to estradiol plus eCG, selection of recipients by the CL diameter, and ET during the breeding season) can improve P/ET until calving and reduce embryo/fetal mortality (**Figure 1**).

Regarding the other factors analyzed, parity did not influence the outcomes of buffalo recipients in the present study, in accordance to another work (35) in which P/ET and pregnancy per treated animal did not differ between nulliparous, primiparous, or multiparous buffaloes submitted to a protocol of fixed time ET. This reinforces the optimization of different animal categories in ET programs in buffaloes.

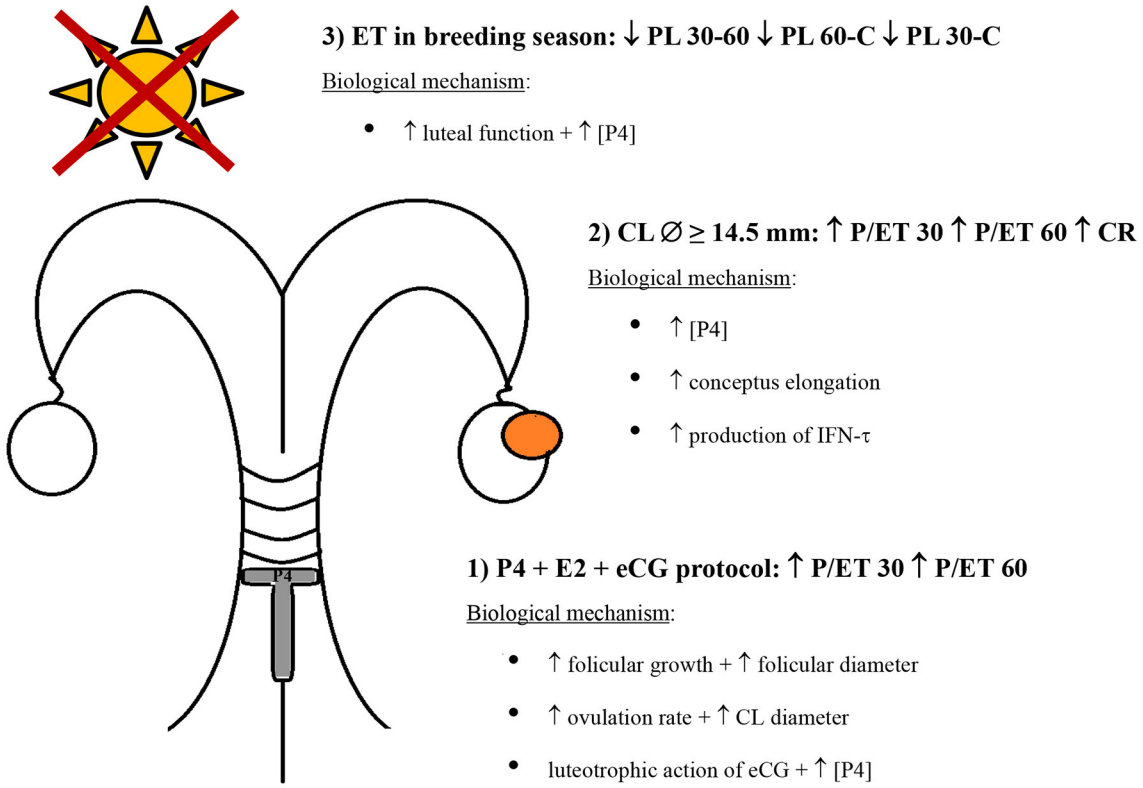

FIGURE 1 | Graphic model of main factors affecting pregnancy per embryo transfer on day 30 (P/ET30), on day 60 (P/ET60), the calving rate (CR), and pregnancy loss from day 30 to 60 (PL 30-60), from day 60 to calving (PL 60-C), and from day 30 to calving (PL 30-C) in buffalo recipients of embryos produced *in vitro*. (1) Effect of the synchronization protocol on P/ET (P4 vs. Ovsynch), (2) effect of the CL diameter on P/ET (≥ 14.5 vs. < 14.5 mm), and (3) effect of season of year on pregnancy loss (breeding season vs. nonbreeding season).

Misra et al. (16) reported that asynchrony ≥ 12 h impaired pregnancy rates in buffalo recipients. Inversely, our data support the possibility of asynchrony between the recipient and the embryo of 1 day (0 and +1) without negative effects on the pregnancy of buffalo recipients, as observed in cattle in which ±24 h did not affect pregnancy rates (36). Associated with this outcome, the day of recipient estrous cycle and the day or stage of embryo development also did not influence our study, in accordance with trials performed in cattle (36, 37) and in buffalo (8, 16). Collectively, the absence of effect of these factors indicates a possible flexibilization in ET schedule allowing the widespread use of this technique in buffalo farms.

In the present study P/ET, the calving rate, or PL was not affected by the type of embryo (fresh vs. vitrified). In cattle, the transfer of fresh embryos is associated to better pregnancy results and lower PL than the transfer of frozen-thawed embryos (36, 37). This can be attributed to injuries caused during cryopreservation. However, in buffaloes, studies comparing these different embryos in contemporary conditions were not found. Nevertheless, our results evidence that vitrification is efficient in buffaloes and can be used to optimize ET programs in this species.

In vitro embryo production outcomes in the current study were, in general, above the average reported in the literature. In previous studies, a low number of total oocytes [1.2 ± 0.07 to 4.1 ± 0.5 (38, 39)], viable oocytes [1.2 ± 0.2 to 1.9 ± 0.3 (38, 40)], blastocyst rate [10–30% (4, 41–43)], P/ET (0–26.9%), and calving rate for fresh [12.5–25.5% (6, 9, 12)] and vitrified embryos [P/ET: 10.5–16.4%, and 10–10.9%, respectively (8, 10, 11)] were reported. These differences can be attributed to several factors, including genetics, nutrition, environment, individual variation in the number of follicles/oocytes, the criterion of oocyte selection, oocyte quality, culture media, use of antioxidants, atmospheric gas, and sire, among others. Corroborating the current study, similar PL [20–33.3% (8, 11)], gestation length [302.3–319.7 days (11, 12)], and weight of calves [27–38.8 kg (8, 10, 12)] were observed. In the present study, no occurrences of large calf syndrome, hydramnio, hydroalantoid, or umbilical cord anomalies were detected. For abnormal postpartum incidence (retained placenta, cervix prolapse, calf rejection, and difficulty of suckling), 12.5 and 5.9% for fresh and vitrified embryos, respectively, were found in the present study. However, no information regarding postpartum problems in buffalo recipients was found in the literature.

CONCLUSION

Our findings indicate that strategies applied to recipients, as synchronization protocols based on progesterone associated to estradiol plus eCG, selection of recipients by the CL diameter, and ET during breeding season, can improve P/ET and calving rates, reducing PL in buffaloes. However, further prospective studies with a larger number of recipients are necessary to confirm these findings.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was conducted according to all ethical care in animal use, according to law number 11,794/ 2008 from Brazilian Constitution (Arouca Law).

AUTHOR CONTRIBUTIONS

WS and MA conceived the study. RM, HB, WS, LG, PB, RD, BG, MR, EB, and RL conducted the experiment. JS analyzed the data. LG wrote the manuscript. PB, BG, RD, MR, and EB critically revised the manuscript. All authors approved the final version of the manuscript.

FUNDING

This work was supported by FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais; Proc APQ-0591-5.04/08).

ACKNOWLEDGMENTS

FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais), Cenatte Embriões LTDA, USP (Universidade de São Paulo), UNINA (Università di Napoli Federico II), and UFMG (Universidade Federal de Minas Gerais).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past co-authorship with the authors BG and PB.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 08 December 2020
doi: 10.3389/fvets.2020.572422

The Effect of Pre-milking Stimulation on Teat Morphological Parameters and Milk Traits in the Italian Water Buffalo

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Water buffaloes (*Bubalus bubalis*) are very sensitive to environmental stimulus before and during milking, and this explains why disrupted milk ejections due to blood oxytocin level instability are frequent in this species. According to the literature, the manual stimulation (MS) of teats before milking promotes oxytocin release and allows milk ejection to start within 2–3 min. However, the pre-milking stimulation of teats is not always part of the milking routine in Italian buffalo farms; moreover, buffaloes with unstable milk let-down are sometimes treated with exogenous oxytocin (OX). Different types of pre-milking stimulation can impact differently on the mammary gland epithelium and structures and, therefore, on milk yield. In this study, we observed the changes in teat morphological traits before and after the application of three types of stimuli, i.e., no manual stimulation (NS), MS, and OX, in buffaloes reared in an Italian dairy farm. In particular, measurements were available for 23 and 21 buffaloes for front and rear teats, respectively. Subsequently, the effect of the pre-milking stimulation type was estimated on teat morphological characteristics and on milk traits recorded after the application of stimuli. The results showed that the teat canal length was shorter ($P < 0.05$) after stimulation in the case of MS and OX compared to NS. Cistern diameter was overall greater for MS and lower for OX. On the contrary, teat wall thickness was greater and lower for OX and MS, respectively. Milk yield and quality (fat, protein, and somatic cell score) were similar across the three types of pre-milking stimulation. In perspective, the impact of these types of pre-milking stimulation should be evaluated on a large scale, and the focus might be put on mammary gland epithelium integrity, mastitis incidence, and other udder health indicators in milk, e.g., electrical conductivity, differential somatic cell count, lactose content, and sodium and chloride concentration.

Keywords: teat morphology, manual stimulation, oxytocin, dairy buffalo, udder health

OPEN ACCESS

Edited by:
Selwyn Arlington Headley, State University of Londrina, Brazil

Reviewed by:
Wendy Beauvais, Purdue University, United States
Tingxian Deng, Institute of Buffalo (CAAS), China

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *14 June 2020*
Accepted: *13 October 2020*
Published: *08 December 2020*

Citation:
Costa A, De Marchi M, Visentin G, Campagna MC, Borghese A and Boselli C (2020) The Effect of Pre-milking Stimulation on Teat Morphological Parameters and Milk Traits in the Italian Water Buffalo. Front. Vet. Sci. 7:572422. doi: 10.3389/fvets.2020.572422

INTRODUCTION

Pre-milking udder stimulation is recommended in dairy species to promote oxytocin release in the bloodstream, reach optimal milk removal and ejection, and thus limit the stress of mammary gland. Literature shows that stimulation of teats by milker's hands seems particularly important to activate the secretory functions (1) in all dairy species. Differently from cattle, buffaloes are

very sensitive to environmental stimulus before and during milking, and therefore disrupted milk ejections due to blood oxytocin level instability are more frequent (2, 3). The blood basal level of oxytocin is 4.8–6.7 ng/L in buffalo, but the concentration may reach 90 ng/L during manual stimulation, with an average of 30 ng/L during milking (2, 3). About 1–2 min of manual stimulation is generally considered appropriate in buffalo and allows milk ejection to start within 2–3 min (1, 4–6). However, in Italy, this procedure is not always performed due to the scarce knowledge of milking personnel and frequent suboptimal milking management (7–9). Considering that complete milk ejection can avoid the apoptosis of mammary gland epithelial cells and is in favor of herd productivity, buffaloes with unstable oxytocin level are sometimes treated with 20 IU of exogenous oxytocin (4). Around 10 min after the injection, the milk starts to flow. This practice is more frequent in primiparous due to higher sensitivity to the environment and greater incidence of milk let-down issues compared to multiparous; generally, around 15–20% of buffaloes in commercial farms are treated (9). Among the disadvantages related to the oxytocin use in buffalo, the risk of drug addiction and progressive resistance to oxytocin stands out; in fact, repeated administrations of this hormone may cause lack of response to normal manual stimulation in the medium to the long term (2, 3, 6).

Mammary gland epithelium and structures may face up changes after pre-milking stimulation. The type of pre-milking stimulation (e.g., none, manual, or oxytocin injection) could affect such morphological and physiological changes to a different extent (1–4). In this study, teat morphological traits were recorded before and after the application of stimuli in water buffaloes (*Bubalus bubalis*) farmed in Italy, and data on milk yield and quality were available in order to estimate the effect of the pre-milking stimulation type.

MATERIALS AND METHODS

Design of the Study

This study was carried out in a commercial Mediterranean water buffalo herd located in the Latium region (Central Italy) from April 2013 to May 2014. Only clinically healthy buffaloes ($n = 38$), with days in milk (DIM) from 6 to 340 and from first to seventh parity were used for this experiment. Buffaloes from fourth parity onwards were grouped. The animals were milked in a herringbone (2 × 5) milking parlor (DeLaval, Tumba, Sweden). The pulsator rate was 60 cycles/min, and the pulsator ratio was 60%; during the experimental milking, the vacuum level was set at 45 kPa. The use of automatic detachment of the milking cluster was activated by the milker after the beginning of alveolar milk emission.

Treatment

There were three pre-milking stimulation types: no manual stimulation (NS), from 1 to 2 min of manual stimulation (MS), and intramuscular injection of 20 IU of exogenous oxytocin (ipofamina 10 IU/ml, TREI—Industria Italiana Integratori S.p.a, Reggio Emilia, Italy) without manual stimulation (OX). During the study, the animals were randomly assigned to each individual treatment at each milking. All pre-milking stimulation types included the cleaning of teats with a wet disposable cotton towel.

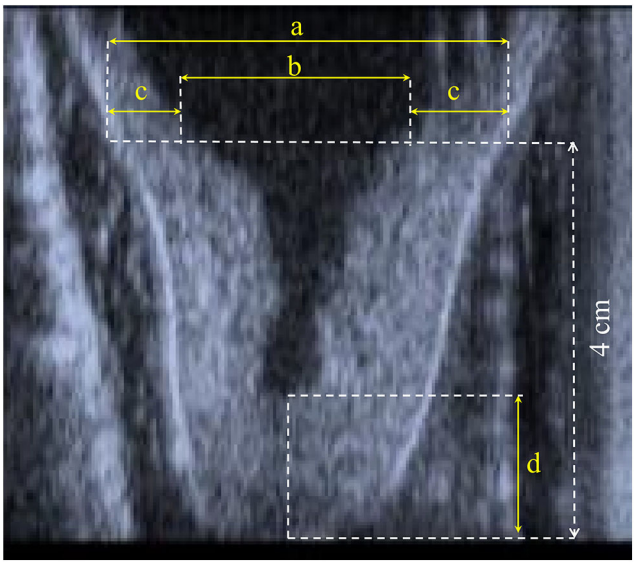

FIGURE 1 | Representation of **(a)** teat diameter, **(b)** diameter of cistern, (**c**) left and right wall thickness, and **(d)** canal length.

Teat Morphological Traits

During the whole trial, the same trained operator, a veterinarian, performed the echography to assess the teat morphological measurements before and after treatment in the same animal. Longitudinal cross-section b-mode ultrasound images (Honda HS 101V; Honda Electronics, Japan, equipped with a 5-MHz linear-array transducer) of the right front and the right rear teats were available (**Figure 1**) to derive the canal length (CL) and, at 4 cm above the teat tip, the teat diameter (TD), the left and the right wall thickness, and the cistern diameter (CD). The total wall thickness (WT) was calculated as the average of the left and the right wall (**Figure 1**). Such traits are conventionally used to study the anatomical characteristics of udder and milkability in dairy species (1–4). The images were recorded with a 5-MHz probe; the teat was put in a cup of hand-warm water, and the ultrasound probe was applied to the outside of the cup by using ultrasound gel (10, 11).

Milk Recording Apparatus

Milk yield (kg) per quarter from the beginning to the end of mechanical milking was recorded during the milking sessions using two Lactocorder® milkmeters (WMB, Balgach, Switzerland). Each milkmeter was vertically attached to every milking place and inserted between the short milk tube and the claw. The measuring chamber consisted of one transmitting electrode and 60 vertically arranged electrodes. The production in each quarter was recorded, and a milk sample (50 ml) representative of the whole milking quarter was collected in tubes (12).

Milk Composition

The milk samples were refrigerated (4°C) and analyzed within 24–36 h from collection at the Experimental Zooprophylactic Institute of Lazio and Tuscany "Mariano Aleandri" (Rome, Italy), the national reference laboratory for dairy product quality in Central Italy. Contents (%) of fat and protein were infrared-predicted from milk spectra using a MilkoScan FT6000 (Foss Analytics, Hillerød, Denmark) calibrated with appropriate buffalo standards, while somatic cell count (SCC, cells/ml) was determined through a flow cytometry technique with a Fossomatic FC (Foss Analytics, Hillerød, Denmark). To achieve a normal distribution of SCC data, the somatic cell score (SCS, units) was calculated as: $SCS = 3 + \log_2 (SCC/100{,}000)$ (13).

Statistical Analyses

The traits of the front and the rear teats were analyzed separately, and at least three measurements (milkings) per animal were guaranteed during the whole experimental trial. After this restriction, 138 and 129 measurements were available for the front (23 animals) and the rear (21 animals) teats, respectively. In particular, for the front teats, 44, 64, and 30 observations were available for NS, MS, and OX, while for the rear teats, 35, 63, and 21 observations were available for NS, MS, and OX. Within each pre-milking stimulation type, a *t*-test with Bonferroni–Holm step-down correction was used in SAS software, v. 9.4 (SAS Institute Inc., Cary, NC, USA) to test the difference between the mean of the trait (CL, TD, WT, or CD of either front or rear teats) recorded before and after the pre-milking stimulation (NS, MS, or OX).

Pearson correlations and their significance were obtained in the same software and were calculated between teat morphological traits separately for the front and the rear teats.

Subsequently, an analysis of variance was used to estimate the effect of the pre-milking stimulation types (NS, MS, or OX) on the teat morphological traits recorded after the pre-milking stimulation and on milk yield and composition (fat and protein content and SCS). The following mixed model was imputed in SAS software, v. 9.4 (SAS Institute Inc., Cary, NC, USA):

$$y_{ijklm} = \mu + P_i + D_j + S_k + (P \times D)_{ij} + (P \times S)_{ik} + (D \times S)_{jk} + B_l + e_{ijklm},$$

where y_{ijklm} is the trait recorded (morphological or milk traits) either in the front or the rear teats, μ is the overall intercept of the model, P_i is the fixed effect of the ith parity of the cow ($i = 1$–4, with class 4 including parities 4–7), D_j is the fixed effect of the jth class of DIM of the cow ($j = 1$–4, with DIM in class 1, 2, 3, and 4, respectively, ≤60, 61–100, 101–150, and ≥151), S_k is the fixed effect of the kth pre-milking stimulation type (NS, MS, or OX), $(P \times D)_{ij}$ is the fixed interaction effect between parity and DIM class, $(P \times S)_{ik}$ is the fixed interaction effect between parity and pre-milking stimulation type, $(D \times S)_{jk}$ is the fixed interaction effect between DIM class and pre-milking stimulation type, B_l is the random effect of the lth buffalo (three or more measurements per buffalo), and e_{ijklm} is the random residual. A multiple comparison of least square means (LSM) for the fixed effects was performed using Bonferroni's test ($P < 0.05$).

TABLE 1 | Mean, coefficient of variation (CV), and range of front ($n = 138$) and rear ($n = 129$) teat measurements of morphological traits before and after pre-milking stimulation.

Trait	Front[a]			Rear[b]		
	Mean	CV, %	Range	Mean	CV, %	Range
Canal length, cm						
Before	2.548	17.91	2.20	2.401	20.27	2.40
After	2.256	24.62	2.70	2.059	28.81	3.60
Teat diameter, cm						
Before	3.137	14.76	2.80	3.193	15.05	2.60
After	3.134	14.84	2.60	3.217	14.59	2.50
Wall thickness, cm						
Before	0.920	27.18	1.20	0.925	25.11	1.05
After	0.891	30.76	1.20	0.896	29.33	1.15
Cistern diameter, cm						
Before	1.297	48.92	2.90	1.343	47.27	3.00
After	1.352	49.27	3.10	1.426	47.09	3.20

[a]*A total of 44, 64, and 30 observations were available for NS, MS, and OX.*
[b]*A total of 35, 63, and 21 observations were available for NS, MS, and OX.*

RESULTS

Before vs. After Pre-milking Stimulation

The descriptive statistics of the teat morphological traits are shown in **Table 1**. During both before and after the pre-milking stimulation, CL was longer in the front than in the rear teats. On the other hand, the opposite was observed for TD and CD. The mean of WT before and after pre-milking stimulation was similar in the front and the rear teats. The coefficient of variation was, in general, the greatest for CD (>47.09%) and the lowest (<15.05%) for TD.

The correlations of the morphological traits of the front teats mirrored those of the rear teats (**Table 2**). Overall, the measures of the same trait before and after pre-milking stimulation were strongly correlated. For example, CD recorded before and after the pre-milking stimulation had a correlation equal to 0.98 and 0.96 for the front and the rear teats, respectively (**Table 2**). Negative associations were observed between CD and CL and between CD and WT, while the correlations between CL and TD and between WT and TD were not significant in both the front and the rear teats. Finally, the correlations observed between WD and CL were moderate.

The means for the trait before and after the pre-milking stimulation and the significance of *t*-test are shown in **Table 3**. In both the front and the rear teats, MS and OX significantly affected CL. In particular, CL was generally shorter after the pre-milking stimulation. However, the differences observed in other traits were not significant at $P < 0.05$.

Comparison of the Types of Pre-milking Stimulation

The effect of the type of pre-milking stimulation was significant for CD and WT of the front teats (**Figure 2**). In particular, the greatest CD and the lowest WT were estimated in the teats that

TABLE 2 | Correlations[a] between morphological traits before and after pre-milking stimulation in the front and the rear teats.

Trait	CL_B, cm	CL_A, cm	TD_B, cm	TD_A, cm	WT_B, cm	WT_A, cm	CD_B, cm
Front							
CL_A, cm	0.57						
TD_B, cm	0.11^{ns}	−0.02^{ns}					
TD_A, cm	0.10^{ns}	−0.07^{ns}	0.94				
WT_B, cm	0.37	0.43	0.07^{ns}	0.05^{ns}			
WT_A, cm	0.29	0.44	0.06^{ns}	0.08^{ns}	0.96		
CD_B, cm	−0.22	−0.35	0.61	0.57	−0.74	−0.75	
CD_A, cm	−0.17*	−0.41	0.56	0.59	−0.75	−0.76	0.98
Rear							
CL_A, cm	0.70						
TD_B, cm	0.08^{ns}	−0.01^{ns}					
TD_A, cm	0.05^{ns}	−0.11^{ns}	0.91				
WT_B, cm	0.28	0.35	0.04^{ns}	−0.05^{ns}			
WT_A, cm	0.21	0.34	0.03^{ns}	−0.01^{ns}	0.95		
CD_B, cm	−0.14^{ns}	−0.26	0.68	0.68	−0.70	−0.68	
CD_A, cm	−0.13^{ns}	−0.33	0.57	0.66	−0.75	−0.76	0.96

[a]*$0.5 \leq P < 0.10$; $^{ns}P \geq 0.10$.*
CL_B, canal length before; CL_A, canal length after; TD_B, teat diameter before; TD_A, teat diameter after; WT_B, wall thickness before; WT_A, wall thickness after; CD_B, cistern diameter before; CD_A, cistern diameter after.

TABLE 3 | Means and SD of teat morphological traits before and after pre-milking stimulation and significance of *t*-test.

Teats	Mean before	SD before	Mean after	SD after	*P*
Front[a]					
No stimulation (NS)					
Cistern diameter, cm	1.302	0.660	1.312	0.661	ns
Teat diameter, cm	3.119	0.404	3.110	0.415	ns
Wall thickness, cm	0.908	0.254	0.899	0.258	ns
Canal length, cm	2.442	0.455	2.352	0.453	ns
Manual stimulation (MS)					
Cistern diameter, cm	1.413	0.643	1.503	0.668	ns
Teat diameter, cm	3.131	0.509	3.125	0.500	ns
Wall thickness, cm	0.859	0.228	0.811	0.258	ns
Canal length, cm	2.606	0.456	2.181	0.587	<0.001
Oxytocin injection (OX)					
Cistern diameter, cm	1.032	0.503	1.086	0.574	ns
Teat diameter, cm	3.179	0.452	3.189	0.458	ns
Wall thickness, cm	1.073	0.236	1.052	0.260	ns
Canal length, cm	2.580	0.448	2.278	0.595	0.016
Rear[b]					
No stimulation					
Cistern diameter, cm	1.344	0.600	1.344	0.599	ns
Teat diameter, cm	3.227	0.372	3.194	0.389	ns
Wall thickness, cm	0.943	0.237	0.925	0.258	ns
Canal length, cm	2.294	0.542	2.174	0.493	ns
Manual stimulation					
Cistern diameter, cm	1.473	0.659	1.609	0.697	ns
Teat diameter, cm	3.125	0.502	3.255	0.514	ns
Wall thickness, cm	0.871	0.221	0.823	0.256	ns
Canal length, cm	2.452	0.417	1.984	0.599	<0.001
Oxytocin injection					
Cistern diameter, cm	1.064	0.546	1.448	0.597	ns
Teat diameter, cm	3.107	0.547	3.166	0.469	ns
Wall thickness, cm	1.021	0.225	1.010	0.240	ns
Canal length, cm	2.419	0.546	2.086	0.673	0.036

ns, not significant at $P \geq 0.05$.
[a]*A total of 44, 64, and 30 observations were available for NS, MS, and OX.*
[b]*A total of 35, 63, and 21 observations were available for NS, MS, and OX.*

were manually stimulated (**Figure 2**). On the other hand, the animals that were treated with oxytocin had front teats with the lowest CD and the greatest WT. Intermediate LSM were estimated for the NS teats.

At both the front and the rear levels, the quantity and the quality of milk were similar among NS, MS, and OX teats (**Table 4**). Despite being not significant, fat content seemed to be greater in NS (front teats) and OX (rear teats); the protein content of the milk of the front teats was the greatest in OX (4.849 %), while in the rear teats it had scarce variation among the three pre-milking stimulation types (**Table 4**). Milk yield was greater in the rear quarters than in the front ones in all the applied treatments, ranging from 0.883 kg (rear teats, NS group) to 1.599 kg (rear teats, OX group). Although milk yield was maximum in general for OX, the LSM of NS, MS, and OX did not differ significantly (**Table 4**).

The interaction effect between the pre-milking stimulation type and parity was relevant for the front ($P < 0.02$) and the rear ($P < 0.08$) teat WT and for the front ($P < 0.03$) and the rear ($P < 0.03$) teat CL and is depicted in **Figure 3**. In particular, the front teat CL of primiparous buffaloes was the lowest in OX (1.56 ± 0.24 cm) and the greatest in NS (2.32 ± 0.30 cm; **Figure 3**). A similar trend was observed for buffaloes in second parity (**Figure 3**). Third-parity buffaloes presented LSM of CL equal to 2.31 ± 0.21, 2.44 ± 0.17, and 2.31 ± 0.23 cm for NS, MS, and OX, respectively. An opposite trend was observed for LSM of buffaloes in fourth parity, i.e., the CL was the lowest in NS (2.01 ± 0.21 cm) and the greatest in OX (2.86 ± 0.27 cm). In both the front and the rear teats, the greatest CL was estimated for fourth-parity buffaloes in the OX group (**Figure 3**).

DISCUSSION

The average measurements (**Table 1**) before the stimuli were comparable to the findings observed in a field study conducted on 18 Mediterranean buffalo in Italy (14), where, using both the front and the rear teats, the authors found average CL, TD, WT, and CD equal to 2.55, 3.27, 0.97, and 1.31 cm, respectively. The mean CL was similar in the front (2.63) and the rear (2.78) positions (14). The average CL (3.10 cm) recorded in 24 Murrah buffaloes was slightly greater compared with the findings observed in the present study (15); in the same study, the authors

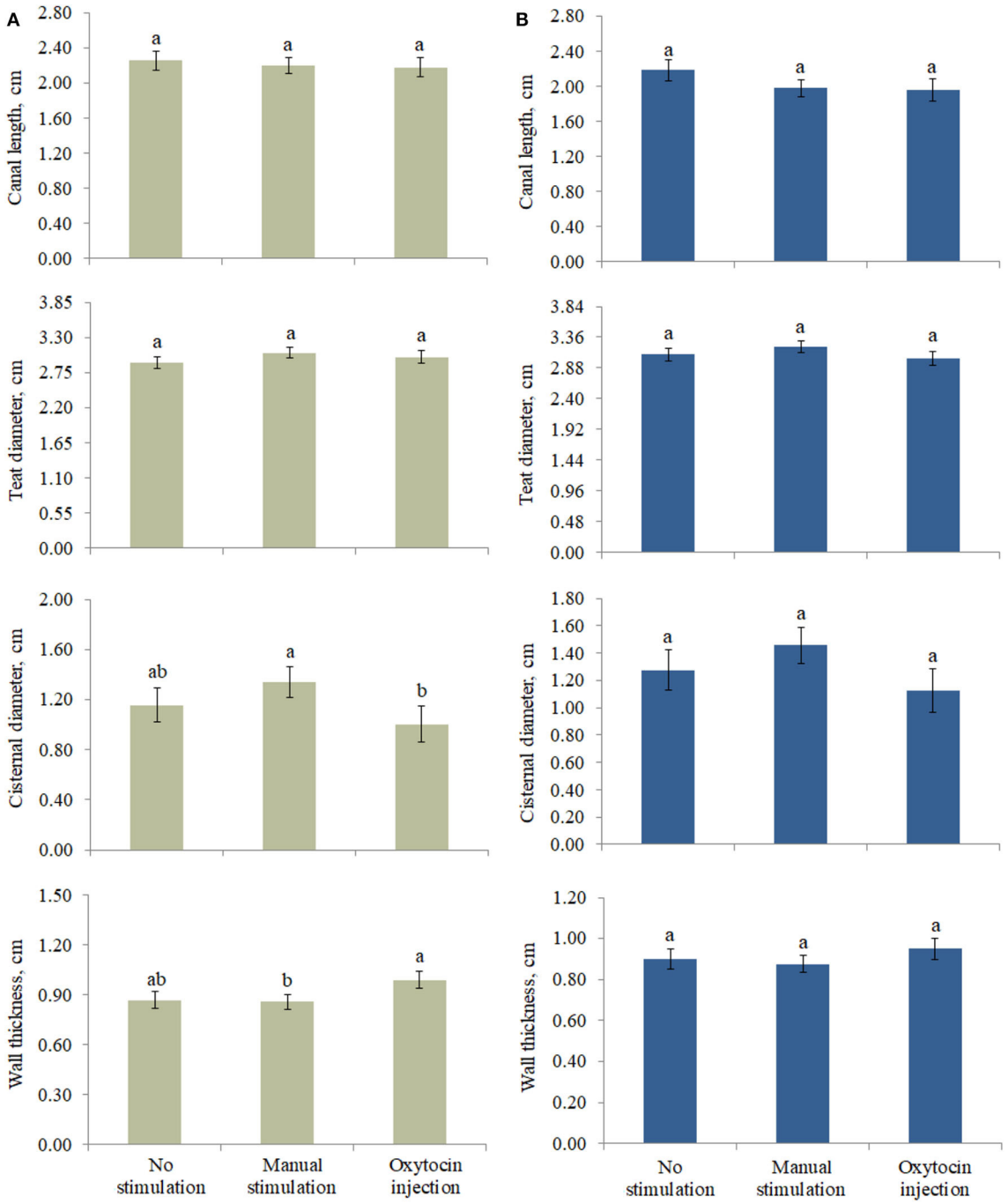

FIGURE 2 | Least square means of **(A)** front and **(B)** rear teat morphological traits for the fixed effect of pre-milking stimulation. Different letters indicate statistical significance ($P < 0.05$), and bars indicate the standard errors of estimates (0.043–0.161). For no stimulation, manual stimulation, and oxytocin injection, the number of observations was equal to 44, 64, and 30 (front teats, **A**) and 35, 63, and 21 (rear teats, **B**), respectively.

observed different CL between the rear (3.7 cm, on average) and the front teats (3.0 cm, on average), but no differences in CL among parities and stages of lactation. In this study, both TD and CD were larger in the rear than in the front teats; this was in agreement with the studies on Murrah buffaloes (15, 16). In fact, a greater cisternal area in the rear (11.84 cm ^{2}) than in the front (9.57 cm^2) quarters was observed. In Mediterranean Italian buffalo (14), the TD was significantly greater in the rear than in the front quarters (6).

The correlations between TD and CL were not significant, which is likely due to the limited sample size and to a weak to null dependency between the features. Within trait, the correlations

suggested a strong (>0.9) dependency between the measures recorded before and after the pre-milking stimulation, except for CL (**Table 2**). In fact, the correlation of CL before and after the application of stimuli was 0.57 in the front teats and 0.70 in the rear teats, suggesting that the type of stimulation may have a different effect on the change of CL. This is also supported by the results of the *t*-test (**Table 3**). In fact, among all traits, only CL was significantly different before and after the stimuli in the case of OX and, more particularly, of MS ($P < 0.001$; **Table 3**). This finding was in line with a recent study conducted on Anatolian buffaloes (11), where significant reductions in CL in the fore and the rear quarters after 3 and 6 min of manual pre-stimulation were observed. In general, according to the literature, a reduction in CL was observed after manual stimulation or the administration of oxytocin (1, 4, 11).

In a study based on 38 dairy cows (17), CL and WT were moderately correlated at the quarter level; the correlation between CL and TD was weak instead.

The animals not stimulated before milking did not present changes in (front and rear) teat morphological traits. With regard to the front teats, the pre-milking stimulation type significantly affected CD and WT, but it is worth to highlight that some differences observed in this study (**Figure 2**) were not significant, which is likely due to the high standard error of estimates. For the same reason, the milk traits estimated for the three pre-milking stimulation types did not differ (**Table 4**); however, the LSM of milk yield was maximum (0.917 ± 0.109 and 1.599 ± 0.142 kg for

TABLE 4 | Least squares means and standard errors (SE) of front and rear teat milk yield and composition traits for the fixed effect of pre-milking stimulation.

Teats	No stimulation (NS)	SE	Manual stimulation (MS)	SE	Oxytocin injection (OX)	SE
Front[a]						
Milk, kg	0.883a	0.099	0.856a	0.091	0.917a	0.109
Fat, %	8.862a	0.465	8.417a	0.448	8.629a	0.623
Protein, %	4.775a	0.119	4.696a	0.116	4.849a	0.153
SCS, units	3.022a	0.320	2.199a	0.307	2.584a	0.433
Rear[b]						
Milk, kg	1.376a	0.129	1.366a	0.118	1.599a	0.142
Fat, %	8.942a	0.461	8.114a	0.465	9.409a	0.627
Protein, %	4.748a	0.101	4.790a	0.102	4.761a	0.136
SCS, units	3.001a	0.359	2.592a	0.342	2.877a	0.489

[a]*A total of 44, 64, and 30 observations were available for NS, MS, and OX.*
[b]*A total of 35, 63, and 21 observations were available for NS, MS, and OX.*
Different letters within a row indicate statistical significance (P < 0.05).

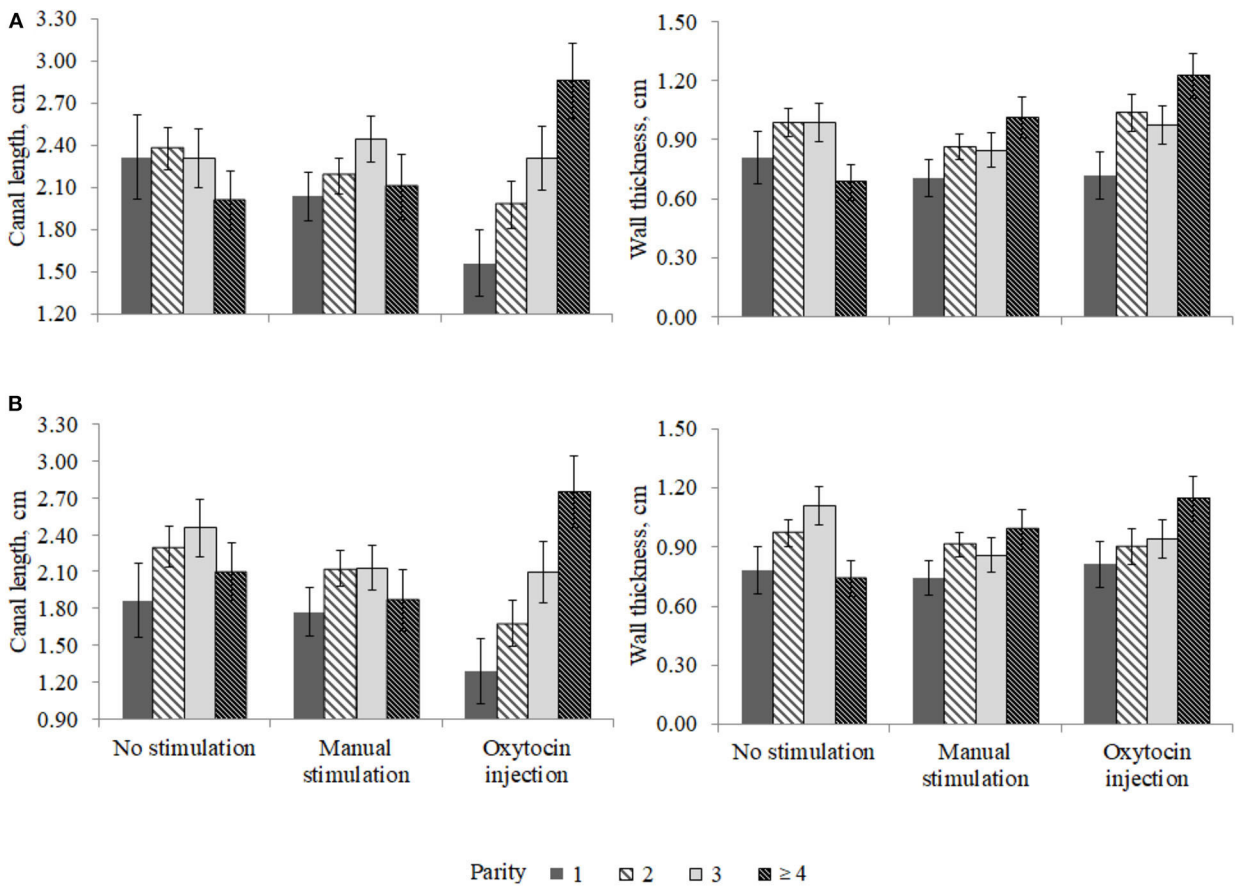

FIGURE 3 | Least square means of **(A)** front and **(B)** rear teat canal length and wall thickness for the fixed effect of the interaction between parity and pre-milking stimulation.

the front and the rear teats, respectively) in OX, supporting the idea that exogenous oxytocin promotes complete milk ejection (4, 6). The rear quarters in buffaloes are slightly larger than the front ones and contain more milk. The approximate ratio is 60:40 (rear/front) as for cattle (16, 17). In support of this, the ratio in our study ranged from 61:39 (NS and MS) to 64:36 (OX).

In a field study (6), the difference in milk yield at the udder level between buffaloes treated and not treated with oxytocin was 10–12%; in the present study, the milk yield of the rear teats in buffaloes treated with oxytocin was 13.95% greater than in animals not treated and 14.57% greater than in the manually stimulated animals (**Table 4**). In the front teats, milk yield was 3.71–6.65% higher if oxytocin was used (**Table 4**). Although not significant, SCS seemed to be worse in NS and the best in MS in both the front and the rear teats. According to the literature (18), the use of oxytocin does not change milk SCS at milking, but no studies have evaluated the effect of prolonged OX on udder health indicators and/or on mastitis incidence (18).

The differences in teat morphological traits (CL and WT) observed among parities (**Figure 3**) may indicates that oxytocin acts differently in the parities considered. Nevertheless, it should be highlighted that the present study was based on a small dataset collected in one farm; thus, the statistical power was relatively low, and some potentially significant differences were not found.

CONCLUSIONS

An adequate udder pre-milking stimulation is usually recommended in dairy species for complete milk removal and optimal milk ejection. In this study, three types of stimuli were investigated: NS, MS, and OX. Both MS and OX caused a reduction in the CL of both the front and the rear teats. Milk yield and quality were similar across the three types of pre-milking stimulation, while significant differences were observed in CD and WT. In particular, the manually stimulated teats showed greater CD and lower WT, while the buffaloes treated with oxytocin had lower CD and greater WT; this was also supported by the correlations estimated in this study. In fact, after the application of stimuli, CD and WT were negatively correlated in both the front (−0.76) and the rear (−0.76) teats. The results suggest that OX may lead to complete milk removal in buffaloes, with apparently no impact on milk quality. Considering that only one farm was involved in this study, these findings may be considered and interpreted with caution. Future experimental trials may be designed in order to include more farms and should evaluate the effect of pre-milking stimulation type on mammary gland epithelial integrity, clinical and subclinical mastitis incidence, and milk indicators of udder health and alveolar permeability, like milk electrical conductivity, differential somatic cell count, lactose content, and sodium and chloride concentration.

DATA AVAILABILITY STATEMENT

The raw data supporting the results and the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical approval was not required for the animal study as per institutional guidelines/local legislation. The owner of the animals used in the study provided written informed consent.

AUTHOR CONTRIBUTIONS

This work was conceived and designed by all the authors. AB and CB were in charge of animal handling and data collection. AC and GV performed the analyses and interpreted the results. The manuscript was mainly written by AC, CB, and AB. MD and MC coordinated the activities and revised the manuscript. GV revised the manuscript and handled the revision process. All the authors contributed to read and approved the final version submitted.

FUNDING

This paper is based on the research project IZSLT 07/19 (G81F19000190001) funded by the Italian Ministry of Health (Rome, Italy).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 10 February 2021
doi: 10.3389/fvets.2020.571654

First Detection of *Listeria monocytogenes* in a Buffalo Aborted Foetus in Campania Region (Southern Italy)

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 11 June 2020
Accepted: 12 August 2020
Published: 10 February 2021

Citation:
Esposito C, Cardillo L, Borriello G, Ascione G, Valvini O, Galiero G and Fusco G (2021) First Detection of Listeria monocytogenes in a Buffalo Aborted Foetus in Campania Region (Southern Italy). Front. Vet. Sci. 7:571654. doi: 10.3389/fvets.2020.571654

Listeria monocytogenes (LM) is the causative agent of listeriosis in both animals and humans, representing one of the most severe food-borne diseases in humans. Out of 13 serotypes, only three (i.e., 1/2a, 1/2b, and 4b) are responsible for 95% of human outbreaks of listeriosis. Ruminants have been hypothesised to represent the main natural reservoir for this pathogen and to be involved in the transmission of *Listeria* to humans. During pregnancy, listeriosis in ruminants cause various reproductive disorders as well as abortion. However, little is known about abortion due to LM in water buffaloes (*Bubalus bubalis*). In this study, we report for the first time the detection of LM in a water buffalo foetus in the region of Campania, Italy. Complete necropsy was performed, and samples and swabs from the abomasum, kidneys, liver, lungs, and spleen were collected. Microbiological and molecular analyses were carried out to detect bacterial, viral, and protozoarian abortive pathogens. The results revealed the presence of LM in the liver, lungs, and abomasum, and no other agent was detected. Isolation was confirmed by biochemical and molecular tests. Molecular serotype characterisation was performed, and serogroup IVb was identified. In conclusion, because of the zoonotic implications of our findings, this report highlights the importance of including LM in the diagnostic panel in cases of bubaline abortion.

Keywords: *Listeria monocytogenes* (LM), serogroup IVb, water buffalo (*Bubalus bubalis*), abortion-spontaneous, bubaline foetus

INTRODUCTION

Listeria monocytogenes (LM) is a food-borne pathogen that causes listeriosis, a zoonotic disease that affects several animal species, including humans. In humans, listeriosis is considered to be a potentially lethal disease, especially in immunocompromised individuals. Notably, this disease is characterised by septicaemia, meningitis, and, more frequently, meningoencephalitis and abortion. Several gastro-intestinal manifestations with fever can also occur (1, 2).

Generally, LM is a ubiquitous, rod-shaped, Gram-positive bacterium that belongs to family Listeriaceae. In total, 20 species of *Listeria* are currently known (3), with LM and *L. ivanovii* being

the only pathogenic species. While *L. ivanovii* are rarely involved in human cases of infection, LM have been recognised as the main species involved in food-borne outbreaks in both animals and humans (2, 4).

In nature, LM strains differ in terms of their epidemiological potential and their ability to cause disease in humans (5). However, several studies have revealed a genetic variation in the isolated populations. This variability is believed to be strongly related to both the microbial load necessary to induce infection and membership to a particular lineage (6, 7). Generally, LM consists of four different lineages (i.e., I, II, III, and IV), with different but overlapping ecological niches, that include 13 serotypes ranging from low to high virulence (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7) (1, 8). Most LM isolates belong to lineages I and II, whose serotypes 1/2a (lineage II) and 1/2b and 4b (lineage I) are associated with human outbreaks (8–10), although strains from lineages III and IV are occasionally associated with human diseases (1, 11, 12).

The main transmission route of this disease is believed to be via the consumption of contaminated food and raw milk. The environment in which food processing occurs is considered an important source of infection for humans (4).

In animals, LM has been isolated from several species, including mammals, birds, and fish (1, 13–15). Ruminants are considered to be the most susceptible species (2) and are believed to be the main reservoir for human infection (16). The main route of infection is believed to be via contaminated feedstuff. Once ingested, the bacterium reaches the target organs (i.e., liver, spleen, brain, and uterus) via the lympho-hematogenous pathway (17). Notably, this disease can be asymptomatic or can present with septicemia and lesions mainly in the central nervous system (meningitis, meningoencephalitis, and rhombencephalitis) (16, 18). Notably, mastitis due to LM is quite rare. However, isolation of the bacterium from raw milk can prove the onset of specific mammary infection (2, 4, 19). LM is also associated with various reproductive disorders, fetal infection, placentitis, and spontaneous abortion, especially during the last trimester of pregnancy (20). Abortion due to LM is widely described in cattle and small ruminants (20, 21), whereas little is known about this condition in water buffalos (*Bubalus bubalis*). Notably, LM has been isolated from meat and milk samples collected from buffaloes (22), and low prevalence has been found in the uterus and in animals with reproductive disorders (23–25).

In this report, we describe the detection and isolation of LM in the liver, abomasum, and lungs of a water buffalo foetus. Molecular characterisation revealed that the isolated strain belonged to serogroup IVb, which includes serotypes 4b, 4d, and 4e. To the best of our knowledge, this is the first report of the detection and isolation of LM in a bubaline aborted foetus in Southern Italy.

METHODS

Anatomopathological and Microbiological Examinations

A bubaline foetus was first submitted to a complete necropsy examination, which was performed within 24 h after death, by postgraduate veterinarians from Istituto Zooprofilattico Sperimentale del Mezzogiorno (IZSM), Portici, Italy, according to standard protocols (26). Surface cauterisation was performed, and organs were incised using sterile scalpels. Approximately 2 cm^2 samples and swabs were collected from the inner parts of the organs. Specimens were processed for bacteriological analysis within 2 h.

Nucleic acid extraction was conducted as follows: 2 mg of tissue samples was first suspended in 2 ml of sterile phosphate-buffered saline in 2 ml tubes, homogenised with glass beads using a TissueLyser (Qiagen, Hilden, Germany) and centrifuged for 5 min at 1,700 × g. Aliquots of 200 μl of the supernatant were then collected, and nucleic acid extraction was performed using a QIAsymphony automated system (Qiagen), processed according to the manufacturer's protocol, eluted in 60 μl, and stored at −80°C until use. All viral, bacterial, and protozoarian abortive pathogens were investigated (**Table 1**).

Notably, LM was determined according to the procedures described by the World Organisation for Animal Health (OIE) in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (15). Swabs were plated in a primary selective enrichment broth, Half Fraser Broth (Oxoid, Rodano, Italy), followed by secondary enrichment in Half Fraser Broth, both incubated at 30 ± 1°C for 24 h. Culture broth was inoculated on selective Oxford Agar (Oxoid) in duplicate and incubated for 24–48 h at 37 ± 1°C. Five colonies, presumed to be *Listeria* spp., were isolated and picked for confirmation. Each colony was plated on Tryptone Soya Yeast Extract Agar (Oxoid) and incubated at 37 ± 1°C for 24 h. The colonies were then submitted to identification on the basis of biochemical macro-methods, Gram stain, catalase, and oxidase tests and hemolysis on Blood Agar (Oxoid), as well as miniaturised biochemical procedures with VITEK 2 Compact (bioMérieux, Lyon, France).

LM Serogrouping by PCR

Bacterial colonies were grown on Ottaviani and Agosti agar (ALOA agar; Oxoid) at 37°C for 24 h. Typical LM colonies (blue-green colour with an opaque halo) were collected for DNA extraction using an InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions. Molecular serogrouping was performed using multiplex PCR by simultaneously amplifying *PrfA*, *lmo0737*, *lmo1118*, *ORF2819*, and *ORF2110* serotype-specific marker genes as well as the *prs* gene, specific for strains of the genus *Listeria* (**Table 2**) (32, 33). The PCR reaction was performed in a final volume of 25 μl and included 2 μl of template, with a DNA concentration of 25 ng/μl and HotStarTaq PCR Master Mix 1X (Promega, Madison, WI, USA), along with $MgCl_2$ (final concentration: 2 mM); primers LMO1118-1 and LMO1118-2 (0.8 μM); primers LMO0737-1, LMO0737-2, ORF2110-1, ORF2110-2, ORF2819-1, and ORF2819-2 (0.4 μM); primers LIP1 and LIP2 (0.2 μM); and primers PRS1 and PRS2 (0.1 μM). The thermal profile consisted of one cycle at 95°C for 15 min for Taq activation and then 35 cycles at 94°C for 30 s, 53°C for 40 s, and 72°C for 1.5 min, followed by a last extension cycle at 72°C for 7 min. Each working session also included the following positive control samples [reported using the Administración Nacional

TABLE 1 | Diagnostic protocols of the abortive pathogens.

Pathogen	Matrix	Test	References
Bovine herpesvirus 1 (BHV-1)	Lungs, liver	PCR	(27)
Bovine herpesvirus 4 (BHV-4)	Lungs, spleen	PCR	(28)
Bovine viral diarrhea virus (BVDV)	Spleen	PCR	(29)
Brucella spp.	Abomasum content, liver, lungs, spleen	Bacteriological	(30)
Campylobacter fetus	Abomasum content, liver	Bacteriological, PCR	(31)
Chlamydophila abortus	Liver, lungs, abomasum content	PCR	(28)
Coxiella burnetii	Liver, lungs, abomasum content	PCR	(28)
Listeria spp.	Abomasum content, liver, lungs	Bacteriological, PCR	(15, 32, 33)
Neospora caninum	Heart	PCR	(28)
Toxoplasma gondii	Heart	PCR	(28)
Leptospira spp.	Kidney	PCR	(28)
Salmonella spp.	Liver	Bacteriological	(34)

TABLE 2 | Primer sets for molecular serogrouping of *Listeria monocytogenes*.

Gene target	Nucleotide sequence
PrfA	LIP1 5′ -GATACAGAAACATCGGTTGGC-3′
	LIP2 5′ -GTGTAATCTTGATGCCATCAGG-3′
Prs	PRS1 5′ -GCTGAAGAGATTGCGAAAGAAG-3′
	PRS2 5′ -CAAAGAAACCTTGGATTTGCGG-3′
Lmo0737	LMO0737-1 5′ -AGGGCTTCAAGGACTTACCC3′
	Lmo0737-2 5′ -ACGATTTCTGCTTGCCATTC-3′
Lmo1118	LMO1118-1 5′ -AGGGGTCTTAAATCCTGGAA-3′
	Lmo1118-2 5′ -CGGCTTGTTCGGCATACTTA-3′
orf 2819	ORF2819-1 5′ -AGCAAAATGCCAAAACTCGT-3′
	ORF2819-2 5′ -CATCACTAAAGCCTCCCATTG-3′
orf2110	ORF2110-1 5′ -AGTGGACAATTGATTGGTGAA-3′
	ORF2110-2 5′ -CATCCATCCCTTACTTTGGAC-3′

de la Seguridad Social (ANSES) reference DNA number]: 00EB248LM (serogroup IIa), 00EB249LM (serogroup IIb), 00EB250LM (serogroup IIc), 00EB254LM (serogroup IVa), 00EB256LM (serogroup IVb), and *Listeria ivanovii* ATCC19119 (serogroup L).

RESULTS

In December 2018, during a routine analysis, a bubaline foetus was brought from a farm located in the province of Caserta, Campania region, Southern Italy, and presented to IZSM in Portici, Naples, Italy, to investigate the cause of abortion. Anamnesis showed that the abortion occurred in the last third of gestation with no prodromal symptoms. A brucellosis eradication campaign, based on serum agglutination and complement fixation tests (30), was performed on the farm in July of the same year, and negative results were obtained for the mother of the aborted foetus.

The foetus was submitted to complete necropsy examination. Organs were *in situ* and normal in shape and size. Severe abdominal and moderate pleural serohematic effusions were observed, and mild pericardial serohematic fluid was found when the pericardium was opened.

Samples and swabs were obtained from the lungs, liver, spleen, abomasum, heart, and kidneys and tested for abortive viruses, bacteria, and protozoa (**Table 1**). Notably, LM was isolated on Oxford Agar from the liver, lungs, and abomasum swabs, and no other agent was detected. The isolated colonies were then submitted for biochemical confirmation using macro-methods and miniaturised procedures (VITEK 2 Compact; bioMérieux). The isolates were then analysed to determine the molecular serogroup. PCR results (**Figure 1**) indicated that this strain could be classified as LM serogroup IVb (corresponding to serotypes 4b, 4d, and 4e).

DISCUSSION

LM is considered one of the most insidious food-borne pathogens that primarily affects individuals at high risk, such as immunocompromised patients, pregnant women, elderly people, and young children, resulting in a mortality rate of around 15–20% (35–37). Human infection occurs as a result of the consumption of contaminated food items, such as milk and dairy products, seafood, and food of animal origin (38). Notably, LM can cross the placental barrier and infect the foetus, causing spontaneous abortion, stillbirth, and even pre-term and neonatal infections in both animals (39) and humans (40). In this study, we report for the first time the detection of LM in a water buffalo foetus that got aborted in December 2019 in the region of Campania, Italy. As described in both cattle and small ruminants, abortion occurred during the last period of gestation (20, 21) and no gross lesions were observed (15, 41).

In ruminants, abortions due to *Listeria* are characterised by a seasonal trend, as they occur mostly in winter or early spring as a result of the higher consumption of contaminated silage, which is recognised as the main route of transmission in these species (20, 21, 42). Furthermore, under certain cold climatic conditions, LM can show a higher pathogenic profile than heat seasons (43).

In humans, from the 13 identified LM serotypes, serotypes 1/2a, 1/2b, and 4d have been identified to cause disease in more

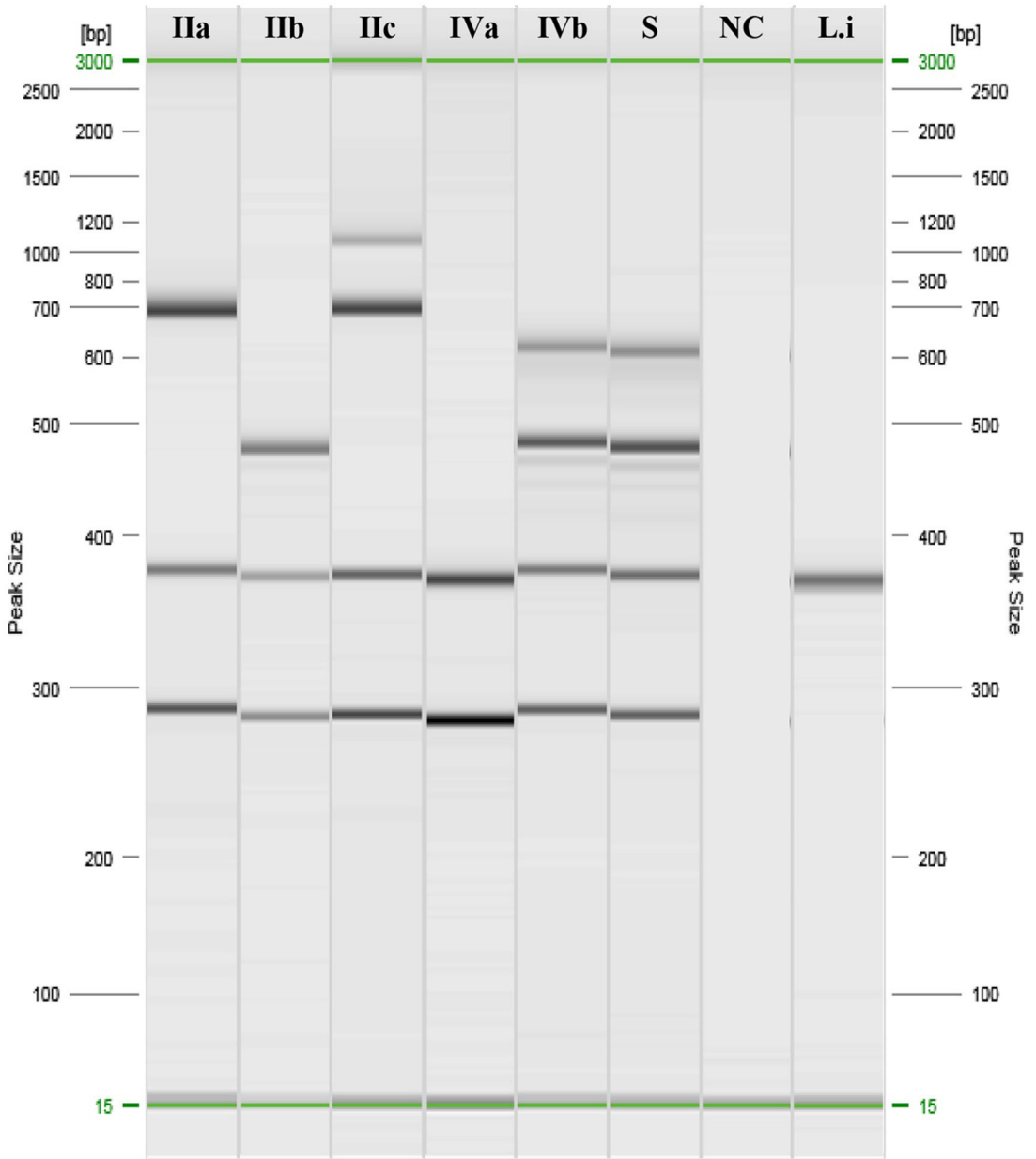

FIGURE 1 | PCR results of *Listeria monocytogenes* serogrouping. IIa: ref. Anses 00EB248LM; IIb: ref. Anses 00EB249LM; IIc: ref. Anses 00EB250LM; IVa: ref. Anses 00EB254LM; IVb: ref. 00EB256LM; S, Sample; NC, Negative control; Li, *Listeria ivanovii* ATCC19119.

than 95% of the cases (44, 45). While serotypes 1/2a and 1/2b have been recognised to cause sporadic illness, serotype 4b is believed to be the main cause of human outbreaks (1, 44). It has been speculated that clones of serotype 4b are more virulent than other strains, although this has not yet been reproduced in infection models (1). Molecular serotyping of LM strains allows the determination of five distinct molecular groups correlated with the serotypes, which allow the correct classification of the most common disease-associated serotypes (i.e., 1/2a, 1/2b, 1/2c, 4a, and 4b) into unique serogroups (33). Notably, the clinical isolates characterised in the present study belong to serogroup IVb, which includes serotypes 4b, 4d, and 4e. This group has been found in several clinical cases from both humans and different farm animal species, such as cattle, sheep, poultry and swine, in different countries (46–48). Serogroup IVb has also been found to be the predominant group among cases of listeriosis in small ruminants in Greece (49), accounting for 68% of all clinical isolates from encephalitis cases and 57% of the isolates from milk samples. Similarly, in Italy, serogroup IVb has been found to be the most represented

among rhombencephalitis-*Listeria*-associated strains from cattle (16). In buffaloes, LM was isolated from meat and milk (22) and sporadic infections in animals with reproductive disorders (23–25), but none of the serotypes has been investigated. However, no abortion due to LM has yet been described in water buffaloes.

The connection between ruminant and human listeriosis is still not clearly understood, as direct transmission between these two species rarely occurs (50). However, it is believed that ruminants act as a reservoir for LM (16). Notably, LM can contaminate food through feces and water (50), and both diseased and asymptomatic animals can discharge LM in their milk and feces, making meat and milk a real risk factor for human infection (21, 51).

In conclusion, considering the zoonotic role of LM and the significance of the findings of this report, it can be concluded that LM serogroup IVb plays a crucial role as a potential abortive agent in water buffaloes and should, therefore, be considered in the diagnostic panel in cases of bubaline abortion.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the experimental Zooprophylactic institutes (IIZZSSMM) are official laboratories designed by the Italian Ministry of Health that are involved in epidemiological surveillance, animal health, food, and feed safety and diagnostics. For this reason, ethics approval was deemed unnecessary in agreement with institutional policy and national regulations. Written informed consent for participation was not obtained from the owners because the study was conducted during institutional activities.

AUTHOR CONTRIBUTIONS

All the authors equally contributed to the study. CE, LC, GB, GA, and OV drafted the manuscript. GG and GF conceded and revised the study. OV and GB conducted the microbiological and biotechnological exams.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Laura Pacifico for the cooperation.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 16 June 2021
doi: 10.3389/fvets.2021.557468

Genetic Parameters for First Lactation and Lifetime Traits of Nili-Ravi Buffaloes

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The data on first lactation and lifetime performance records of 501 Nili-Ravi were collected for a period from 1983 to 2017 (35 years) maintained at ICAR-Central Institute for Research on Buffaloes, Sub-Campus, Nabha, Punjab. The data were analyzed to calculate heritability, genetic and phenotypic correlation for first lactation traits, viz., Age at First Calving (AFC), First Lactation Total Milk Yield (FLTMY), First Lactation Standard (305 days or less) Milk Yield (FLSMY), First Peak Milk Yield (FPY), First Lactation Length (FLL), First Dry Period (FDP), First Service Period (FSP) and First Calving Interval (FCI), Herd Life (HL), Productive Life (PL), Productive Days (PD), Unproductive Days (UD), Breeding Efficiency (BE), Total Lifetime Milk Yield (Total LTMY), Standard Lifetime Milk Yield (Standard LTMY), Milk Yield Per Day of Productive Life (MY/PL), Milk Yield Per Day of Productive Days (MY/PD), and Milk Yield Per Day of Herd Life (MY/HL). For estimation of variance component and heritability separately for each trait, the uni-trait animal model was equipped, whereas to estimate genetic and phenotypic correlations between traits, bi-trait animal models were fitted. The estimates of heritability for production and reproduction traits of Nili-Ravi were medium, i.e., 0.365 ± 0.087, 0.353 ± 0.071, 0.318 ± 0.082, 0.354 ± 0.076, and 0.362 ± 0.086 for FLSMY, FDP, FSP, FCI, and AFC, respectively. The estimates of heritability were low, i.e., 0.062 ± 0.088, 0.123 ± 0.090, 0.158 ± 0.090, 0.155 ± 0.091, and 0.129 ± 0.091 for HL, PL, PD, Total LTMY, and Standard LTMY and high, i.e., 0.669 ± 0.096 for BE. Genetic correlation for FLTMY was high with FLL (0.710 ± 0.103), and genetic correlation of FLTMY was high and positive with HL, Total LTMY, MY/PL, and MY/PD while low and positive with PL. Genetic correlation of AFC was low and negative with PL, PD, UD, BE, Total LTMY, Standard LTMY, MY/PL, and MY/PD and negative with MY/HL. Significant positive phenotypic association of FPY was seen with FLTMY, FLSMY, FLL, AFC, HL, Total LTMY, and Standard LTMY. Higher heritability of first lactation traits especially FPY suggests sufficient additive genetic variability, which can be exploited under selection and breeding policy in order to improve overall performance of Nili-Ravi buffaloes.

Keywords: buffalo, Nili-ravi, genetic, phenotypic, traits, association, heritability

OPEN ACCESS

Edited by:
Selwyn Arlington Headley, State University of Londrina, Brazil

Reviewed by:
Fabyano Fonseca Silva, Universidade Federal de Viçosa, Brazil
Anupama Mukherjee, Indian Council of Agricultural Research (ICAR), India

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *30 April 2020*
Accepted: *05 May 2021*
Published: *16 June 2021*

Citation:
Tamboli P, Bharadwaj A, Chaurasiya A, Bangar YC and Jerome A (2021) Genetic Parameters for First Lactation and Lifetime Traits of Nili-Ravi Buffaloes. Front. Vet. Sci. 8:557468. doi: 10.3389/fvets.2021.557468

INTRODUCTION

India is ranked first in the world with 176.3 million tons of milk production, and most of the production (49%) was contributed by buffaloes. The per capita availability of milk up to 375 g/day was achieved in recent time (1). Buffaloes are present almost in all parts of the country with varying population density, the majority being concentrated in the North and Western parts. Nili-Ravi, a buffalo breed, is found in the state of Punjab and largely distributed in the belt of Ravi River, having its concentration in Amritsar, Tarn Taran, and Ferozepur districts (2). Lifetime performance of animal especially depends on different production and reproduction traits of earlier lactations, which determine genetic worth of the buffaloes. Hence, the lifetime traits can be improved by the selection of animals on the basis of traits of earlier lactations through correlated response. Assessment of genetic progress attained by the selection programs gives an idea about advantages of breeding program and to incorporate required adjustments.

The traits having high heritability can be efficiently improved through appropriate selective breeding. The heritability provides an opportunity to forecast the outcome of breeding strategy. High heritability observed for the First Lactation Total Milk Yield (FLTMY) and First Lactation Standard (305 days or less) Milk Yield (FLSMY) in Nili-Ravi (3) indicates that there is enough scope of the traits to respond in the next generation. Reduction in Age at First Calving (AFC) is associated with improvement in the lifetime milk yield, herd life, productive life, and productive days in buffaloes (4). For economizing milk production, it is desirable to decrease AFC (5). Early first calving of animals would result in longer productive life (6). Thus, dairymen have a considerable concern in the relationship of different production and reproduction parameters with lifetime traits. Framing for profitable selection and breeding systems requires knowledge of genetic and phenotypic parameters of early and lifetime performance traits to enhance genetic potential of buffaloes. Information regarding genetic and phenotypic relationship between first lactation and lifetime traits in Nili-Ravi buffaloes is scarce. Therefore, the present study was designed to evaluate heritability, genetic and phenotypic correlation for first lactation and lifetime performance traits in Nili-Ravi buffaloes.

MATERIALS AND METHODS

Study Location, Collection of Data, and Its Classification

The present study was carried out on the performance records of Nili-Ravi buffaloes ($n = 501$) maintained at ICAR-Central Institute for Research on Buffaloes, Sub-Campus, Nabha, Punjab. The data on first lactation and lifetime performance records of study animals that remained in the herd for at least three lactations and disposed were collected from the history sheet of animal for a period from 1983 to 2017. The data for all the buffaloes born and calved at the farms were recorded up to the date of death/culling. The animals having minimum lactation length >150 days and production >1,000 kg for first lactation were considered. Incomplete and abnormal records due to abortion, stillbirth, chronic illness, etc., were excluded.

Traits analyzed in our study were first lactation traits, viz., AFC in months, FLTMY in kg, FLSMY in kg, First Peak Milk Yield (FPY) in kg/day, First Lactation Length (FLL) in days, First Dry Period (FDP) in days, First Service Period (FSP) in days, First Calving Interval (FCI) in days, Lifetime performance traits such as Herd Life (HL) in days, Productive Life (PL) in days, Productive Days (PD) in days, Unproductive Days (UD) in days, Breeding Efficiency (BE) in %, Total Lifetime Milk Yield (Total LTMY) in kg, Standard Lifetime Milk Yield (Standard LTMY) in kg, Milk Yield Per Day of Productive Life (MY/PL) in kg/day, Milk Yield Per Day of Productive Days (MY/PD) in kg/day, and Milk Yield Per Day of Herd Life (MY/HL) in kg/day.

The herd life was considered the duration from birth to disposal of animal. Productive life was defined as total days from date of first calving to date of last dry or date of disposal if animal is in lactation. Productive days were the sum of the number of days in milk in different lactations in the same herd. Unproductive days were the sum of dry periods in different lactations in the same herd. Lifetime milk yield was defined as total amount of milk produced by a buffalo from the initiation of first lactation until last day in milk in the herd. It was taken for those animals that remained in the herd for at least three lactations. Milk yield per day of productive life was measured as lifetime milk yield divided by the productive life. Milk yield per day of productive days was measured as lifetime milk yield divided by the productive days. Milk yield per day of herd life was measured as lifetime milk yield divided by herd life (7, 8).

Statistical Analysis

The data were analyzed to calculate heritability, genetic and phenotypic correlation for first lactation, and lifetime traits. Data were initially analyzed by least squares analysis model (SPSS Statistics 20.0 software) to identify the significant fixed effects. The least squares model for AFC included season of birth (Level 4) and period of birth (Level 6) as fixed effects. Fixed effects for first lactation and lifetime traits were AFC (Level 6), season of first calving (Level 4), and Period of first calving (Level 6). Lactation completed (Level 5) was also included along with other fixed effects for lifetime traits. The statistical significance was tested at 5% and 1% level. Only significant effects under least squares analysis for each trait were included under univariate animal model for estimating heritability and associations between AFC, first lactation traits, and lifetime productivity (9, 10).

The estimates of variance components and genetic parameters were obtained *via* Average Information-Restricted Maximum Likelihood (REML) using Wombat software (11). The convergence was assumed if the difference in log likelihood function between consecutive iterations was lower than 5×10^{-4}.

The univariate animal model consisting of significant fixed effects and random animal effects was used in this study as follows:

$$y = X\beta + Zu + e$$

where y is the vector of records for different traits (FLTMY, Total LTMY, etc.)

X is the incidence matrices assigning observations to fixed effects

β is the vector of fixed effects

Z is the incidence matrices assigning observations to random effects

u is the vector of random animal effects

e is the vector of random residual effects (error) distributed normally with mean zero and variance σ^2

Furthermore, bivariate analysis was performed between each pair of the first lactation and lifetime traits to estimate the genetic and phenotypic correlations (12). The significance of phenotypic correlation was tested using "*t*-test" at 5 or 1% level.

RESULTS

Heritability Estimates for First Lactation Production and Reproduction Traits

Heritability estimates are helpful for the prediction of genetic response and accuracy in selection (5). The estimates of heritability for production and reproduction (**Table 1**) traits of Nili-Ravi were medium for FLSMY (0.365 ± 0.087), FDP (0.353 ± 0.071), FSP (0.318 ± 0.082), FCI (0.354 ± 0.076), and AFC (0.362 ± 0.086); moderate for FLTMY (0.439 ± 0.091) and FLL (0.546 ± 0.065); and high for FPY (0.640 ± 0.112).

Heritability Estimates for Lifetime Performance Traits

The heritability estimates for lifetime performance traits of Nili-Ravi are presented in **Table 2**. In Nili-Ravi, the estimates of heritability were low for HL (0.062 ± 0.088), PL (0.123 ± 0.090), PD (0.158 ± 0.090), Total LTMY (0.155 ± 0.091), and Standard LTMY (0.129 ± 0.091); medium (0.2–0.4) for UD (0.228 ± 0.089), MY/PL (0.354 ± 0.094), MY/PD (0.231 ± 0.086), and MY/HL (0.261±0.089); and high for BE (0.669 ± 0.096).

Estimates of Genetic Correlation for First Lactation Performance Traits

Estimates of genetic correlation among production and reproduction traits in Nili-Ravi are presented in **Table 1**. High and positive correlation for FLTMY with FLL (0.710 ± 0.103), medium and positive with FPY (0.361 ± 0.153), and moderate and positive with FLSMY (0.421 ± 0.149) was observed. Genetic correlation of FLSMY was medium and positive with FPY (0.562 ± 0.144) while low and positive with FLL (0.026 ± 0.133). The correlation between FPY and FLL (−0.309 ± 0.150) was medium and negative. Estimates of genetic correlation were low and positive for AFC with FSP (0.065 ± 0.261), FCI (0.098 ± 0.233), and FDP (0.091 ± 0.230). Genetic correlation of FDP was moderate and positive with FCI (0.536 ± 0.128) and FSP (0.581 ± 0.134). The correlation between FSP and FCI (0.963 ± 0.020) was high and positive.

The genetic correlation between first lactation production and reproduction traits for Nili-Ravi is presented in **Table 1**. In Nili-Ravi, estimates of genetic correlation (**Table 1**) were low and

TABLE 1 | Heritability (diagonal), genetic (above diagonal), and phenotypic (below diagonal) correlation among first lactation traits.

Traits	FLTMY (kg)	FLSMY (kg)	FPY (kg/day)	FLL (days)	AFC (months)	FDP (days)	FSP (days)	FCI (days)
FLTMY (kg)	**0.439 ± 0.091**	0.421 ± 0.149	0.361 ± 0.153	0.710 ± 0.103	0.164 ± 0.208	−0.377 ± 0.185	0.359 ± 0.218	0.319 ± 0.198
FLSMY (kg)	0.687 ± 0.026**	**0.365 ± 0.087**	0.562 ± 0.144	0.026 ± 0.133	0.442 ± 0.227	0.491 ± 0.240	0.483 ± 0.252	0.445 ± 0.226
FPY (kg/day)	0.459 ± 0.040**	0.573 ± 0.035**	**0.640 ± 0.112**	−0.309 ± 0.150	0.201 ± 0.193	−0.089 ± 0.182	−0.215 ± 0.216	−0.356 ± 0.201
FLL (days)	0.564 ± 0.026**	0.131 ± 0.035**	0.108 ± 0.041*	**0.546 ± 0.065**	0.022 ± 0.158	−0.357 ± 0.151	0.512 ± 0.131	0.597 ± 0.116
AFC (months)	0.142 ± 0.042**	0.210 ± 0.041**	0.107 ± 0.046*	0.008 ± 0.036	**0.362 ± 0.086**	0.091 ± 0.230	0.065 ± 0.261	0.098 ± 0.233
FDP (days)	−0.259 ± 0.037**	0.017 ± 0.038	−0.025 ± 0.043	−0.214 ± 0.03**	0.023 ± 0.038	**0.353 ± 0.071**	0.581 ± 0.134	0.536 ± 0.128
FSP (days)	0.179 ± 0.041**	0.098 ± 0.041*	−0.042 ± 0.046	0.488 ± 0.027**	0.009 ± 0.041	0.728 ± 0.021**	**0.318 ± 0.082**	0.963 ± 0.020
FCI days)	0.188 ± 0.039**	0.110 ± 0.039*	−0.100 ± 0.044*	0.543 ± 0.026**	0.025 ± 0.039	0.704 ± 0.021**	0.981 ± 0.003**	**0.354 ± 0.076**

*Significant at *p < 0.05, **p < 0.01.*

AFC, Age at First Calving; FLTMY, First Lactation Total Milk Yield; FLSMY, First Lactation Standard (305 days or less) Milk Yield; FPY, First Peak Milk Yield; FLL, First Lactation Length; FDP, First Dry Period; FSP, First Service Period; FCI, First Calving Interval.

Bold values represent Heritability

TABLE 2 | Heritability (diagonal), genetic (above diagonal), and phenotypic (below diagonal) correlation among lifetime traits.

Traits	HL (days)	PL (days)	PD (days)	UD (days)	BE (%)	Total LTMY (kg)	Standard LTMY (kg)	MY/PL (kg/day)	MY/PD (kg/day)	MY/HL (kg/day)
HL (days)	**0.062 ± 0.088**	0.304 ± 0.197	0.611 ± 0.219	−0.439 ± 3.447	−0.229 ± 0.471	0.750 ± 0.239	0.529 ± 0.261	0.529 ± 0.504	0.298 ± 0.403	0.483 ± 0.332
PL (days)	0.893 ± 0.014**	**0.123 ± 0.090**	0.887 ± 0.103	0.562 ± 0.217	−0.305 ± 0.322	0.676 ± 0.200	0.815 ± 0.191	−0.355 ± 0.594	−0.384 ± 0.906	0.578 ± 0.251
PD (days)	0.890 ± 0.014**	0.969 ± 0.008**	**0.158 ± 0.090**	0.117 ± 0.191	−0.126 ± 0.284	0.868 ± 0.130	0.867 ± 0.154	−0.005 ± 53500	−0.233 ± 0.866	0.717 ± 0.195
UD (days)	0.704 ± 0.026**	0.847 ± 0.018**	0.689 ± 0.027**	**0.228 ± 0.089**	−0.431 ± 0.215	−0.101 ± 2.241	0.202 ± 0.249	−0.755 ± 0.334	−0.409 ± 0.471	−0.042 ± 1.552
BE (%)	−0.046 ± 0.044	−0.143 ± 0.044**	−0.066 ± 0.044	−0.278 ± 0.042**	**0.669 ± 0.096**	0.063 ± 0.279	0.059 ± 0.278	0.272 ± 0.173	0.045 ± 0.190	0.120 ± 0.209
Total LTMY (kg)	0.865 ± 0.017**	0.900 ± 0.015**	0.938 ± 0.011**	0.621 ± 0.030**	0.041 ± 0.045	**0.155 ± 0.091**	0.936 ± 0.069	0.352 ± 0.266	0.142 ± 0.216	0.788 ± 0.131
Standard LTMY (kg)	0.844 ± 0.018**	0.903 ± 0.014**	0.923 ± 0.013**	0.662 ± 0.029**	0.080 ± 0.044	0.985 ± 0.005**	**0.129 ± 0.091**	0.116 ± 0.223	0.018 ± 0.050	0.759 ± 0.148
MY/PL (kg/day)	0.295 ± 0.041**	0.157 ± 0.044**	0.274 ± 0.041**	−0.131 ± 0.044**	0.242 ± 0.042**	0.477 ± 0.035**	0.440 ± 0.036**	**0.354 ± 0.094**	0.703 ± 0.129	0.207 ± 0.198
MY/PD (kg/day)	0.393 ± 0.037**	0.308 ± 0.039**	0.328 ± 0.038**	0.199 ± 0.041**	0.087 ± 0.043*	0.554 ± 0.030**	0.548 ± 0.030**	0.638 ± 0.022**	**0.231 ± 0.086**	−0.019 ± 68.352
MY/HL (kg/day)	0.478 ± 0.031**	0.568 ± 0.028**	0.629 ± 0.024**	0.313 ± 0.038**	0.074 ± 0.044	0.710 ± 0.017**	0.700 ± 0.018**	0.417 ± 0.032**	0.416 ± 0.030**	**0.261 ± 0.089**

*Significant at *$p < 0.05$, **$p < 0.01$.*

HL, Herd Life; PL, Productive Life; PD, Productive Days; UD, Unproductive Days; BE, Breeding Efficiency; Total LTMY, Total Lifetime Milk Yield; Standard LTMY, Standard Lifetime Milk Yield; MY/PL, Milk Yield Per Day of Productive Life; MY/PD, Milk Yield Per Day of Productive Days; MY/HL, Milk Yield Per Day of Herd Life.

Bold values represent Heritability

positive for FLTMY with AFC (0.164 ± 0.208) and medium and negative with FDP but positive with FSP and FCI. Moderate and positive genetic correlation of FLSMY was noticed with AFC, FDP, FSP, and FCI. Genetic correlation of FPY was low and negative with FDP and medium and positive with AFC but negative with FSP and FCI. The genetic correlation of FLL was low and positive with AFC, medium and negative with FDP, while moderate and positive with FSP and FCI.

Genetic Correlation Among Lifetime Traits

In Nili-Ravi, estimates of genetic correlation (**Table 2**) of HL was high and positive with PD and Total LTMY; moderate and positive with Standard LTMY, MY/PL, and MY/HL but negative with UD; medium and positive with MY/PD and PL and negative with BE. Genetic correlation of PL was high and positive with PD, Total LTMY, and Standard LTMY; moderate and positive with UD and MY/HL; and medium and negative with BE, MY/PL, and MY/PD. The correlation of PD was high and positive with Total LTMY, Standard LTMY, and MY/HL; medium and negative with MY/PD; low and positive with UD but negative with BE and MY/PL. The correlation of UD was medium and positive with Standard LTMY; low and negative with Total LTMY and MY/HL; moderate and negative with BE and MY/PD; high and negative with MY/PL. The genetic correlation of BE was low and positive with Total LTMY, Standard LTMY, MY/PD, and MY/HL; medium and positive with MY/PL. **Table 2** elucidates that the genetic correlation of Total LTMY was high and positive with Standard LTMY (0.936 ± 0.069) and MY/HL (0.788 ± 0.131); medium and positive with MY/PL; low and positive with MY/PD. The genetic correlation of Standard LTMY was low and positive with MY/PL and MY/PD but high and positive with MY/HL. The correlation of MY/PL was medium and positive with MY/HL but high and positive with MY/PD. The genetic correlation between MY/PD and MY/HL was low and negative.

Genetic Correlation Between First Lactation Performance and Lifetime Traits

The genetic correlation estimates between first lactation production and reproduction traits with lifetime traits of Nili-Ravi are presented in **Table 3**. It can be inferred from **Table 3** that the genetic correlation of FLTMY was high and positive with HL, Total LTMY, MY/PL, and MY/PD; moderate and positive with Standard LTMY; medium and positive with PD and MY/HL. Hence, selection for one trait simultaneously leads to genetic gain in the other parameters, as the genetic correlation between the traits is higher and positive.

But the correlation of FLTMY was medium and negative with UD and BE while low and positive with PL. The FLSMY was associated positively with HL, PL, PD, UD, Total LTMY, Standard LTMY, MY/PL, and MY/PD but negatively with BE and MY/HL. The genetic correlation of FPY showed that it was low and positive with HL; low and negative with Total LTMY; medium and negative with PL, PD, UD, Standard LTMY, and MY/HL; medium and positive with BE; moderate and positive with MY/PL; high and positive with MY/PD. The FLL was correlated positively with HL, PL, PD, Total LTMY, Standard

TABLE 3 | Genetic correlation between first lactation and lifetime traits in Nili-Ravi buffaloes.

Traits	FLTMY (kg)	FLSMY (kg)	FPY (kg/day)	FLL (days)	AFC (months)	FDP (days)	FSP (days)	FCI (days)
HL (days)	0.887 ± 0.683	0.323 ± 0.578	0.074 ± 0.382	0.834 ± 0.579	0.330 ± 0.525	−0.383 ± 0.649	0.382 ± 0.635	0.425 ± 0.599
PL (days)	0.141 ± 0.384	0.083 ± 0.373	−0.396 ± 0.419	0.603 ± 0.336	−0.108 ± 0.374	0.234 ± 0.370	0.673 ± 0.444	0.746 ± 0.424
PD (days)	0.374 ± 0.334	0.015 ± 0.178	−0.332 ± 0.357	0.777 ± 0.291	−0.024 ± 0.266	−0.137 ± 0.348	0.480 ± 0.384	0.585 ± 0.364
UD (days)	−0.365 ± 0.273	0.151 ± 0.322	−0.258 ± 0.266	−0.096 ± 0.207	−0.188 ± 0.288	0.748 ± 0.241	0.588 ± 0.290	0.556 ± 0.266
BE (%)	−0.228 ± 0.164	−0.366 ± 0.184	0.244 ± 0.159	−0.228 ± 0.115	−0.114 ± 0.178	−0.645 ± 0.133	−0.833 ± 0.125	−0.761 ± 0.113
Total LTMY (kg)	0.674 ± 0.325	0.099 ± 0.271	−0.047 ± 1.370	0.870 ± 0.316	−0.106 ± 0.361	−0.423 ± 0.359	0.339 ± 0.396	0.423 ± 0.370
Standard LTMY (kg)	0.483 ± 0.359	0.179 ± 0.322	−0.036 ± 1.002	0.727 ± 0.350	−0.164 ± 0.400	−0.186 ± 0.384	0.416 ± 0.453	0.498 ± 0.426
MY/PL (kg/day)	0.653 ± 0.161	0.085 ± 0.177	0.458 ± 0.175	0.258 ± 0.167	−0.082 ± 0.273	−0.653 ± 0.192	−0.304 ± 0.243	−0.328 ± 0.223
MY/PD (kg/day)	0.603 ± 0.209	0.327 ± 0.227	0.664 ± 0.195	0.036 ± 0.198	−0.182 ± 0.314	−0.288 ± 0.263	−0.091 ± 0.287	−0.215 ± 0.277
MY/HL (kg/day)	0.363 ± 0.221	−0.051 ± 0.785	−0.258 ± 0.290	0.684 ± 0.201	−0.261 ± 0.265	−0.316 ± 0.255	0.210 ± 0.296	0.346 ± 0.275

*For abbreviations, refer to the legends of **Tables 1**, **2**.*

LTMY, MY/HL, MY/PD, and MY/PL while negatively with UD and BE.

Perusal of **Table 3** revealed that in Nili-Ravi, the genetic correlation of AFC was low and negative with PL, PD, UD, BE, Total LTMY, Standard LTMY, MY/PL, and MY/PD; medium and positive with HL; medium and negative with MY/HL. The genetic correlation of FDP was high and positive with UD; medium and positive with PL while moderate and negative with Total LTMY; high and negative with BE and MY/PL; low and negative with PD and Standard LTMY; medium and negative with HL, MY/PD, and MY/HL. The FSP was positively correlated with HL PL PD, UD Total LTMY, Standard LTMY, and MY/HL, whereas it was negative with MY/PL, MY/PD, and BE. The positive association of FCI was found with HL, PL, PD, UD, Total LTMY, Standard LTMY, and MY/HL whereas negative with BE, MY/PL, and MY/PD.

Phenotypic Correlation for First Lactation Performance Traits

It is apparent from **Table 1** that in Nili-Ravi, estimates of phenotypic correlation was high and positive for FLTMY with FLSMY (0.687 ± 0.026); moderate and positive with FPY (0.459 ± 0.040) and FLL (0.564 ± 0.026). The correlation of FLSMY was highly significant ($p \leq 0.01$) and positive with FPY (0.573 ± 0.035) and FLL (0.131 ± 0.035). The association between FPY and FLL was significant ($p \leq 0.05$) and positive. Estimates of phenotypic correlation were low and positive for AFC with FDP, FSP, and FCI. Phenotypic association of FDP was high and positive with FCI (0.704 ± 0.021) and FSP (0.728 ± 0.021). The correlation between FSP and FCI (0.981 ± 0.003) was also high and positive.

Perusal of **Table 1** indicates that in Nili-Ravi, estimates of phenotypic correlation were highly significant ($p \leq 0.01$) and positive for FLTMY with AFC, FSP, and FCI but highly significant ($p \leq 0.01$) and negative with FDP. The correlation of FLSMY was significant ($p \leq 0.05$) and positive with AFC, FSP, and FCI but non-significant ($p > 0.05$) with FDP. Phenotypic correlation of FPY was significant ($p \leq 0.05$) and positive with AFC (0.107 ± 0.046) but negative with FCI. The correlation of FLL was highly significant ($p \leq 0.01$) and negative with FDP but positive with FSP and FCI. No relationship between FLL and AFC was found.

Phenotypic Correlation Among Lifetime Traits

It is obvious from **Table 2** that estimates of phenotypic correlation in Nili-Ravi for HL were high and positive with PL (0.893 ± 0.014), PD (0.890 ± 0.014), UD (0.704 ± 0.026), Total LTMY (0.865 ± 0.017), and Standard LTMY (0.844 ± 0.018); moderate and positive with MY/HL; medium and positive with MY/PD and MY/PL whereas low and negative with BE. The correlation of PL was high and positive with PD, UD, Total LTMY, and Standard LTMY; moderate and positive with MY/HL, medium and positive with MY/PD; low and positive with MY/PL but low and negative with BE. Positive correlation of PD was observed with UD, Total LTMY, Standard LTMY, MY/HL, MY/PL, and MY/PD whereas negative with BE. The association of UD was high and positive with Total LTMY and Standard LTMY; medium and positive with MY/HL; low and positive with MY/PD but low and negative with MY/PL; moderate and negative with BE. Low and positive phenotypic correlation of BE was observed with Total LTMY, Standard LTMY, MY/PD, and MY/HL while medium and positive with MY/PL. **Table 2** represents that the correlation of Total LTMY was high and positive with Standard LTMY and MY/HL; moderate and positive with MY/PD and MY/PL. High and positive phenotypic association of Standard LTMY was found with MY/HL (0.700 ± 0.018) but moderate and positive with MY/PL and MY/PD. The correlation of MY/PL was high and positive with MY/PD; moderate and positive with MY/HL. Moderate and positive correlation was seen between MY/PD and MY/HL.

Phenotypic Correlation Between First Lactation Performance and Lifetime Traits

The phenotypic correlation estimates between first lactation performance and lifetime traits are shown in **Table 4** for Nili-Ravi buffaloes. The phenotypic correlation between first lactation production and lifetime traits in Nili-Ravi is presented in **Table 4**. The association of FLTMY and FLSMY was significant ($p \leq 0.05$) and positive with HL, PD, Total LTMY, Standard LTMY, MY/PL, MY/PD, and MY/HL while negative with UD and BE. The correlation of FPY was highly significant ($p \leq 0.01$) and positive with Total LTMY (0.208 ± 0.046), Standard LTMY (0.206 ± 0.046), MY/PL, MY/PD, and MY/HL; significant ($p \leq 0.05$) and positive with HL (0.113 ± 0.047). The FLL was associated positively with PL, PD, Total LTMY, Standard LTMY, and MY/HL and negatively with UD, BE, and MY/PD.

It is clear from **Table 4** that in Nili-Ravi, the phenotypic correlation of AFC was positive with HL, MY/PL, and MY/PD and negative with UD, BE, and MY/HL. The correlation of FDP was highly significant ($p \leq 0.01$) and positive with PL and UD but negative with BE, MY/HL, and MY/PL. The phenotypic association of FSP was highly significant ($p \leq 0.01$) and positive with PL, PD, and UD and negative with BE, MY/PL, and MY/PD. The FCI was associated positively with HL, PL, PD, UD, Total LTMY, Standard LTMY, and MY/HL and negatively with BE, MY/PL, and MY/PD.

DISCUSSION

Moderate to high estimates of heritability for various traits show sufficient additive genetic variance for the traits that could be used for selection. In this study, higher estimates of heritability for FPY indicate the extreme reliance of the trait upon the growth rate and other structural traits that commonly have high heritability. Hence, FPY should be given more weightage while selection as the trait is highly heritable and responds in the next generation, which simultaneously also leads to improvement in the lifetime performance. In accordance with our findings, Chaudhari (8) recorded medium heritability for FSP and FCI in Nili-Ravi. Medium heritability for AFC was reported by Kumar

TABLE 4 | Phenotypic correlation between first lactation and lifetime traits in Nili-Ravi buffaloes.

Traits	FLTMY (kg)	FLSMY (kg)	FPY (kg/day)	FLL (days)	AFC (months)	FDP (days)	FSP (days)	FCI (days)
HL (days)	0.143 ±0.043**	0.087 ±0.042*	0.113 ±0.047*	0.080 ±0.036	0.135 ±0.041**	−0.028 ±0.039	0.052 ±0.041	0.034 ±0.040
PL (days)	0.052 ±0.044	0.087 ±0.043*	0.039 ±0.048	0.094 ±0.036*	−0.076 ±0.042	0.127 ±0.038**	0.186 ±0.041**	0.178 ±0.039**
PD (days)	0.147 ±0.043**	0.127 ±0.042**	0.048 ±0.048	0.189 ±0.036**	−0.055 ±0.042	0.011 ±0.039	0.147 ±0.041**	0.147 ±0.040**
UD (days)	−0.165 ±0.043**	−0.018 ±0.042	0.012 ±0.048	−0.131 ±0.036**	−0.104 ±0.042*	0.349 ±0.035**	0.230 ±0.039**	0.204 ±0.038**
BE (%)	−0.114 ±0.044*	−0.127 ±0.043**	0.073 ±0.049	−0.249 ±0.034**	−0.137 ±0.043**	−0.568 ±0.032**	−0.688 ±0.029**	−0.670 ±0.028**
Total LTMY (kg)	0.292 ±0.041**	0.257 ±0.041**	0.208 ±0.046**	0.169 ±0.036**	−0.048 ±0.043	−0.066 ±0.039	0.067 ±0.042	0.066 ±0.040
Standard LTMY (kg)	0.226 ±0.042**	0.267 ±0.041**	0.206 ±0.046**	0.104 ±0.037*	−0.060 ±0.043	−0.032 ±0.039	0.049 ±0.042	0.048 ±0.040
MY/PL (kg/day)	0.509 ±0.034**	0.404 ±0.036**	0.428 ±0.042**	0.042 ±0.037	0.052 ±0.043	−0.289 ±0.036**	−0.202 ±0.041**	−0.218 ±0.040**
MY/PD (kg/day)	0.415 ±0.036**	0.439 ±0.034**	0.512 ±0.038**	−0.140 ±0.036**	0.023 ±0.042	−0.070 ±0.038	−0.120 ±0.041**	−0.161 ±0.039**
MY/HL (kg/day)	0.316 ±0.039**	0.272 ±0.039**	0.118 ±0.046**	0.248 ±0.035**	−0.127 ±0.041**	−0.097 ±0.038*	0.067 ±0.041	0.098 ±0.040*

*Significant at *$p < 0.05$, **$p < 0.01$.*
*For abbreviations, refer to the legends of **Tables 1, 2**.*

et al. (13) in Murrah, Singh and Yadav (14) in Nili-Ravi, and Kumar et al. (15) in cattle. In contrast, high heritability was observed for the FLTMY and FLSMY in Nili-Ravi (3). Also, Chaudhari (8) reported low heritability for FLTMY, FLSMY, FLL, and FDP; medium for FPY; and high for AFC in Nili-Ravi. Higher estimates of heritability, i.e., 0.63 and 0.82, for AFC were observed by Singh and Gurnani (16) in Karan Swiss and Karan Fries cattle, respectively. While lower heritability for AFC was estimated as 0.11 in Mehsana buffaloes by Parmar et al. (5) and 0.03 by Brzakova et al. (17) in Holstein Friesian. The reason for higher heritability of first lactation traits in Nili-Ravi breed under our investigation could be attributed to less inbreeding and more genetic variation. Similar to our results, low heritability for LTMY, PL, and HL was noticed by Bashir et al. (18) in Nili-Ravi buffaloes. Singh et al. (19) also reported low heritability for LTMY in Nili-Ravi. Contrary to our finding, heritability estimated for Total LTMY, PL, and HL were medium while low for UD in Nili-Ravi buffaloes (8). Ali et al. (4) also reported medium heritability estimates of LTMY and HL while moderat for PL.

Strong genetic correlation exists between these reproductive traits; hence, any one of the traits can be used to evaluate reproductive status. A highly positive association reveals the increase in FSP; there is subsequent increment in FDP and FCI as the gestation period and lactation length almost remain constant. Similar to our results, the genetic correlation of FLTMY was positive with FLSMY, FPY, and FLL in Nili-Ravi buffaloes (8). Similarly, highly positive genetic correlation of FDP with FSP and FCI was reported by Godara (20) in Murrah. Also, Chaudhari (8) reported a positive genetic correlation of AFC with FLTMY, FLSMY, FPY, FLL, FDP, FSP, and FCI; FDP with FSP and FCI. The FLSMY had medium positive genetic correlation with FSP and FCI. Sachdeva and Gurnani (21) also reported a positive genetic correlation between FLTMY and FCI in dairy animals. However, our results differ from the findings of Chaudhari (8) who revealed a negative correlation of FLSMY with FDP, and Patil et al. (22) noticed negative relationships of AFC with FSP, FLTMY, and FPY, while AFC was positively correlated with FCI in Murrah.

Highly positive association of productive days with lifetime milk yield confesses the traits are influenced by the same genes. A high correlation among different traits indicates concurrent progression of the traits through selection and genetic improvement to enhance genetic potential and establish herd of superior animals. In agreement to our findings, highly positive genetic correlations among lifetime traits were reported by Kaushik et al. (23) and Singh et al. (24) in Hariana and Karan Swiss cattle, respectively. Ambhore et al. (25) observed genetic correlations of Total LTMY with other lifetime traits, viz., HL and PL as high and positive, whereas between Total LTMY and BE was positive and low in cows. Chaudhari (8) also indicated a positive genetic correlation for Total LTMY with HL and PL; PL with HL and UD. On the contrary, UD with HL and Total LTMY was high and positive in Nili-Ravi buffaloes.

AFC is one of the essential traits for bringing improvement in milk production. It considerably affects the productive life and lifetime milk production of an animal. Reduction in AFC is desirable for reducing rearing cost of heifer and for economizing

milk production (5). In agreement with our findings, Chaudhari (8) reported that AFC had positive genetic correlations with HL but negative correlation with UD; FLTMY had moderate to high and positive genetic correlation with Total LTMY and PL. Low and negative genetic correlation of FDP with Total LTMY and HL but positive with UD was also seen. The genetic correlation of FSP was positive with UD and HL and that of FCI was low and positive with Total LTMY, PL, HL, and UD in Nili-Ravi buffaloes. The results are also in line with findings of Ali et al. (4) where significant ($p \leq 0.05$) and negative genetic correlation of AFC with Total LTMY, PL, HL, and PD was noticed. Negative and significant ($p \leq 0.05$) genetic correlation between AFC and LTMY has also been reported by Lin and Allaire (26), Bhatia (27), and Hussain (28). Contradictory to our results, Chaudhari (8) reported AFC had low and positive genetic correlations with Total LTMY and PL; low and negative genetic correlation of FDP with PL; genetic correlation of FSP was low and negative with Total LTMY and PL. Genetic correlations of FLTMY with Total LTMY and FDP with BE were low in Murrah buffaloes while high in Nili-Ravi, which may be attributed to variations in genetic makeup of the two breeds.

Our results of positive phenotypic correlation of FLTMY with FLSMY, FPY, and FLL agreed with Chaudhari (8). The phenotypic correlation of FLSMY was high and significant ($p \leq 0.05$) with FPY and FLL. Similar findings were reported between FLTMY and FLL by Singh and Raut (29) and for FLTMY and FLL in dairy cattle by Samoilo (30). The negative association between FLTMY and FDP could be due to an increase in lactation length or vice versa. Likewise, Patil et al. (22) reported that phenotypic correlation of AFC was significant ($p \leq 0.05$) and positive with FPY; FSP was non-significantly ($p > 0.05$) negative with FPY; FCI was highly significant ($p \leq 0.01$) and positive with FLTMY but non-significantly ($p > 0.05$) negative with FPY. In contrast, Dev et al. (31) observed positive phenotypic correlations of FPY with other early performance traits, viz., FSP and FCI. Negative phenotypic correlation of FLSMY with FDP was reported by Chaudhari (8).

High correlation among different lifetime traits illustrates that selection based on any one of these traits would lead to a positive correlated response in other traits. In line with our results, Chaudhari (8) noticed that the phenotypic correlation of Total LTMY was highly significant ($p \leq 0.01$) with HL, PL, and UD; and of PL was highly significant ($p \leq 0.01$) with HL and UD. The association observed between UD and HL was also highly significant ($p \leq 0.01$) in Nili-Ravi buffaloes. Our results also agreed with that of Ambhore et al. (32), where phenotypic correlations of Total LTMY with HL and PL were positive and significant ($p \leq 0.05$) while with BE was positive and non-significant ($p > 0.05$).

Chaudhari (8) in Nili-Ravi buffaloes reported that phenotypic correlations of AFC were positive with HL; low and negative with UD; FLTMY was positive with PL, HL, and Total LTMY; FDP was low and negative with HL and Total LTMY. The correlations of FSP with HL, PL, UD, and Total LTMY were low and positive; and FCI was low and positive with HL, PL, UD, and Total LTMY. Supportive findings are also reported by Larson et al. (33), Dutt and Taneja (34), and Ali et al. (4) who observed a negative phenotypic correlation between AFC and Total LTMY. On the contrary, Chaudhari (8) found positive phenotypic correlations of AFC with PL, UD, and Total LTMY; negative correlations of FDP with PL and UD. Keeping in view the results, the selection on lifetime performance is not practically feasible due to long generation interval; it is desirable to select the animals on the basis of performance of earlier lactations rather than traits expressed later in life (8).

In conclusion, heritability estimates were high for FPY and BE; moderate for FLTMY and FLL; medium for FLSMY, FDP, FSP, FCI, AFC, and UD. Higher heritability of the traits indicates sufficient additive genetic variance; hence, these traits should be given more attention for selection and breeding strategies to increase genetic gain in the herd. The genetic correlation in Nili-Ravi for AFC was positive with HL and negative with lifetime traits (PL, PD, UD, Total LTMY, Standard LTMY, and BE). Positive genetic correlation for AFC with HL indicates that a delay in first calving offered more time to these animals to stay longer in the herd in unproductive stage until first calving, which might increase herd life of the animals. Negative genetic association of AFC with lifetime traits suggests an animal owner or breeder to make efforts for the reduction of AFC up to a certain extent by means of improved management systems (breeding, feeding, reproduction and housing management, etc.) to enhance production, reproduction, and lifetime performance of the livestock population.

DATA AVAILABILITY STATEMENT

Data shall be made available as requested by the corresponding authors, without undue reservation.

ETHICS STATEMENT

The study was reviewed and approved by the Institute Animal Ethics Committee, ICAR-Central Institute for Research on Buffaloes, Hisar, India.

AUTHOR CONTRIBUTIONS

PT contributed to the collection of data, data curation, and drafting of the manuscript. AB contributed to the design of the experiment. AC contributed to the collection of data and curation. YB contributed to the statistical analysis. AJ contributed to the interpretation of results and revision of the manuscript. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The Authors thank the Director, ICAR-Central Institute for Research on Buffaloes, for providing necessary support for this research. The authors duly acknowledge the support of Officer In-charge, ICAR-Central Institute for Research on Buffaloes, Sub-Campus, Nabha, Punjab, during this study.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 18 June 2021
doi: 10.3389/fvets.2021.593871

Genome Wide Prediction, Mapping and Development of Genomic Resources of Mastitis Associated Genes in Water Buffalo

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OPEN ACCESS

Edited by:
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Reviewed by:
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National Bureau of Animal Genetic Resources (NBAGR), India
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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: 11 August 2020
Accepted: 30 April 2021
Published: 18 June 2021

Citation:
Jaiswal S, Jagannadham J, Kumari J, Iquebal MA, Gurjar AKS, Nayan V, Angadi UB, Kumar S, Kumar R, Datta TK, Rai A and Kumar D (2021) Genome Wide Prediction, Mapping and Development of Genomic Resources of Mastitis Associated Genes in Water Buffalo. Front. Vet. Sci. 8:593871. doi: 10.3389/fvets.2021.593871

Water buffalo (*Bubalus bubalis*) are an important animal resource that contributes milk, meat, leather, dairy products, and power for plowing and transport. However, mastitis, a bacterial disease affecting milk production and reproduction efficiency, is most prevalent in populations having intensive selection for higher milk yield, especially where the inbreeding level is also high. Climate change and poor hygiene management practices further complicate the issue. The management of this disease faces major challenges, like antibiotic resistance, maximum residue level, horizontal gene transfer, and limited success in resistance breeding. Bovine mastitis genome wide association studies have had limited success due to breed differences, sample sizes, and minor allele frequency, lowering the power to detect the diseases associated with SNPs. In this work, we focused on the application of targeted gene panels (TGPs) in screening for candidate gene association analysis, and how this approach overcomes the limitation of genome wide association studies. This work will facilitate the targeted sequencing of buffalo genomic regions with high depth coverage required to mine the extremely rare variants potentially associated with buffalo mastitis. Although the whole genome assembly of water buffalo is available, neither mastitis genes are predicted nor TGP in the form of web-genomic resources are available for future variant mining and association studies. Out of the 129 mastitis associated genes of cattle, 101 were completely mapped on the buffalo genome to make TGP. This further helped in identifying rare variants in water buffalo. Eighty-five genes were validated in the buffalo gene expression atlas, with the RNA-Seq data of 50 tissues. The functions of 97 genes were predicted, revealing 225 pathways. The mastitis proteins were used for protein-protein interaction network analysis to obtain additional cross-talking proteins. A total of 1,306 SNPs and 152 indels were identified from 101 genes. Water Buffalo-MSTdb was developed with *3-tier architecture* to retrieve mastitis associated genes having genomic coordinates with chromosomal details for TGP sequencing for mining of minor alleles for further

association studies. Lastly, a web-genomic resource was made available to mine variants of targeted gene panels in buffalo for mastitis resistance breeding in an endeavor to ensure improved productivity and the reproductive efficiency of water buffalo.

Keywords: GWAS, mammary gland, markers, mastitis, TGP, water buffalo

INTRODUCTION

Water buffalo (*Bubalus bubalis*) have been domesticated for more than 5,000 years, are also known as "Black Gold" due to their economic value. Its contribution to the Indian GDP in terms of milk, meat, horns, hides, leather, dairy products like cream, butter, yogurt, and cheese, power, plowing, and transporting people and crops have made buffalo an important animal resource in rural areas. India ranks first in world milk production due to the largest bovine population, where buffalo contributes 55%. The estimated world population of water buffalo is 208 million which is spread across over 77 countries in five continents with a major population (96%) in Asia (1). Water buffalo is an efficient converter of poor-quality forages into high quality milk and meat, making it a very valuable genetic resource for countries having "Low External-Input System" (2). Its efficiency of rumen fermentation (3) and nitrogen utilization (4) ability make it the animal of preference. However, intense selection for dairy productivity often results in increased levels of inbreeding, due to the limited number of bulls in the gene pool. Furthermore, climate change with a rising temperature humidity index (THI) has induced mastitis and many other diseases in major dairy animals including buffalo like retained placenta, metritis, ovarian cysts, claw diseases, milk fever, ketosis, and displaced abomasum (5–7).

Mastitis infections cause inflammation of the mammary gland and udder tissue, which is one of the most complex, costlier endemic diseases of dairy animals. It occurs as an immune response to invasion by a group of bacterial species in the teat canal. This may also occur due to chemical, mechanical, or thermal injury to the udder. This disease causes huge economic loss due to lesser milk production, premature culling, veterinary costs, and the risk of drug residues (8). Thus, there is a need to reduce mastitis to increase profitability and health (9).

Reduced milk production in buffalo mastitis costs US$8.80 per lactation (10). The direct loss happens due to a reduction in milk yield (70%), milk discard after treatment (9%), cost of veterinary services (7%), and premature culling (14%). There is also an indirect loss by adverse reproductive efficiency because of an imbalance in luteinizing hormone (LH) and estradiol that leads to failure of ovulation (11). In India, a total annual loss due to mastitis (clinical and subclinical) in buffalo has been estimated at US$526 million (10). Both clinical and subclinical mastitis is associated with mammary inflammation, a difference seen in clinical detectable and undetectable changes, respectively. With clinical mastitis, clinical visible signs are accompanied in the milk or mammary parenchyma. In subclinical mastitis conditions, a specific diagnostic test is performed (12).

Antibiotic treatment is effective in only 60% of cases of mastitis, which is due to an increase in b-lactamase producing organisms (10). The indiscriminate use of antibiotics has also led to a rise in resistant bacterial strains in Indian buffalo (13). All these are of global concern which is highly alarming due to the rise in AMR (antimicrobial resistance) and HGT (Horizontal Gene Transfer) of ARG (Antibiotic Resistance Genes) (14). The traditional breeding approach for the reduction of mastitis has not been effective due to its low heritability and its unfavorable genetic correlation with milk yield (15).

Recent studies on GWAS of mastitis disease in bovines have revealed that breed difference, sample size, and minor allele frequency, may lower the power to detect the disease-associated SNPs (16). Though universal NGS array based GWAS and genomic selection have been successful in animal breeding for better genetic gain of productivity traits, mastitis resistance breeding has still had limited success. GWAS has a major limitation in that it implicates the entire genome in disease predisposition where most association signals reflect variants/genes with no direct biological relevance to the disease (17). A control group of animals may contain unexposed individuals with susceptible genotypes. In such situations, a candidate gene approach tends to have greater statistical power than GWAS (18). GWAS can miss data on intron retention, which works in disease resistance, as reported in water buffalo (19). The success of the candidate gene approach depends on the correct choice of targeted gene panel (TGP) for SNP discovery, focusing on disease associated pathways in the investigation (20).

In genome assisted selection, the candidate gene approach using TGP and GWAS complement each other, rather than being contradictory. TGP can be used pragmatically in dissecting the genetics of complex disease by the mining of extremely low frequency alleles required in case-control association analysis. This ensures that minor novel alleles are not missed, which are not present in the prefabricated SNP array/chip (21, 22). TGP approach offers multifold advantages like sequencing at higher depth (500–1000X coverage) than the whole genome/exome sequencing, allowing the discovery of extremely rare variants which are potentially the most valuable alleles for association studies (23, 24). It is rapid and cost effective, especially in the case of association studies where the sample size is limited and mining of causative mutations in a single assay is imperative (25). Variant mining of TGP can be done either by direct amplicon sequencing (if TGP <50) or by target enrichment by magnetic beads followed by NGS data generation (26).

Post-GWAS, prioritization of candidate genes for mastitis resistance breeding in cattle has been reported (27). A computational approach has been reported to identify the candidate genes for disease association studies (28). A genomic resource database for cattle candidate genes and genetic markers for milk production and mastitis has also been developed (29). However, there is no targeted gene panel genomic resource of

water buffalo for mastitis association studies. Recently available WGA with chromosome wise genomic data can be used for the development of genomic resources cataloging TGP. Since *taurine* and *babuline* genomes have extensively conserved milk and mastitis associated genes, comparative genomics approaches can quickly enrich buffalo genomic resource development.

The present work aims to develop a targeted gene panel of mastitis genes in water buffalo. It anticipates the prediction of mastitis associated candidate genes in the water buffalo genome using publically available bovine mastitis genes. It also aims to validate the RNA-Seq library in water buffalo, along with predicting biochemical pathways mediating this disease through the protein-protein network analysis. We present a web-genomic resource that is required for variant mining for future association studies of mastitis disease.

MATERIALS AND METHODS

Mining and Mapping of Bovine Mastitis Associated Genes

An intensive literature search was carried out covering >40 literature across water buffalo (*Bubalus bubalis*) and cattle (*Bos taurus*) genes. Distinct terms associated with mastitis, *namely*, mastitis resistance, tolerance, and traits association were used as keywords for this search. These records were compiled and filtered to remove duplicates to finally fetch the genes associated with mastitis for further analyses in the present study.

Earlier studies on buffalo mastitis genes were reported even before the availability of the *de novo* genome assembly but then chromosomal number and position in the physical map were not known. By applying the NGS-based variant mining from TGP in a given buffalo population, the targeted genes or region of interest can be mapped. To accomplish this, the entire set of genes reported in *Bubalus bubalis* was downloaded from NCBI (30). Prior to further analyses, these were subjected to manual curation based on the organism specific term "*Bubalus bubalis.*" The finally filtered genes were used to map mastitis associated genes to obtain their genomic coordinates in the chromosome-wise genome assembly. In-house Perl scripts were used to map, based on gene symbol and its *alias*. Based on these parameters, mapping was accomplished for genes indicating features like gene symbol, gene ID, description, chromosome number, chromosome start and end location, strand orientation, and exon count.

Validation of Predicted TGP Genes in the Water Buffalo Gene Expression Atlas

Since genes in TGP panels are computationally predicted using cattle genome, their expressional reliability was validated in the RNA-Seq data of water buffalo available in the gene expression atlas of 50 different tissues (31). This validation was done using an in-house generated Perl script.

Functional Prediction of Mastitis Associated Candidate Genes

The function and pathway enrichment analysis of genes were performed using PANTHER (32) and DAVID v6.8 (Database for Annotation, Visualization, and Integrated Discovery) available at https://david.ncifcrf.gov/ (33, 34). It utilizes the Kyoto Encyclopedia of Genes and Genomes (KEGG) for pathway analysis and Gene Ontology (GO) analyses for functional prediction.

Annotation tool DAVID had *Bubalus bubalis* specific 91 genes. Since, in bovines, mastitis-associated genes are highly conserved, further analysis was done by taking advantage of this using cattle (*Bos taurus*) in PANTHER (http://pantherdb.org/data/). KEGG Mapper (Version 4.1), a mapping tool was used to reconstruct pathways (35). KEGG Orthology (KO) annotation was retrieved for key candidate genes and pathway reconstruction was performed specific to the *Bubalus bubalis* species.

Prediction of Cross-Talking Proteins by Protein-Protein Network Analysis

The list of genome-based candidate genes was further enriched by computational prediction of mastitis disease associated proteins (DAP) using protein-protein interaction (PPI) analysis. The amino acid sequences of mastitis associated genes were retrieved from NCBI, using advanced search options with the keywords "mastitis" and organism "*Bubalus bubalis*" (36). The cross talk of candidate gene proteins with additional predicted putative candidate gene proteins were predicted computationally by PPI analysis using the STRING database (Version 11.0; https://string-db.org/) (37). This database scores and integrates the various sources of PPI after collecting these from the public domain and complements the information with computational predictions. STRING database aims at a comprehensive and objective global network, considering both, physical as well as functional interactions. Proteins are represented as nodes and the interactions among them are pictured as edges in the network. Furthermore, Cytoscape (Version 3.7.1) (38) was used to visualize the PPI network. To get the most inter-connected nodes from the constructed PPI network complex, additional Cytoscape plugins (MCODE and CentiScape) were used. To predict the extended interaction among proteins, the retrieved number of proteins was increased up to 100. All these interactions were predicted specific to *Bubalus bubalis* species.

Evaluation of Mastitis TGP Based Variant Mining (SNP and Indel)

RNA-Seq data of mammary gland tissues (ERR315636) and Refseq of water buffalo (GCA_003121395.1) were downloaded from NCBI. The data was processed by FastQC (39) to check the quality. After the quality was checked low-quality reads and adapters were removed using the Trimmomatic tool V. 0.36 (40) by selecting the parameters of Phred quality score ≥20, Minimum reads length = 25 and Leading:4, Trailing:4 and Sliding-window:4:20. Filtered pair-end reads were aligned to the reference (genome assembly of water buffalo) using Burrows-Wheeler Aligner (bwa-0.7.17) tool (41, 42). The Sequence Alignment Map (SAM) file was converted to Binary Alignment Map (BAM) using SAMtools-1.9 (43) followed by sorting of BAM file. Variant calling analysis for analyzing genotype likelihoods was done with mpileup function with BCFtools 1.9 (44). SNPs

were filtered at $p < 0.05$, read depth (d) ≥10, quality depth (Q) ≥30, MQ (minimum root mean square mapping quality) ≥40, and flanking sequence length 50. Genomic coordinates of SNPs and indels from the VCF file were mapped with coordinates of 101 genes using in-house generated shell scripting.

Development of Web Resource: Water Buffalo-MSTdb (*WBMSTDb*)

Water Buffalo Mastitis Database (*WBMSTDb*) is an online relational database that catalogs information of bovine mastitis associated genes, their information along with the functions and annotations, predicted genes and their functions by close and other interaction PPI networks and *in-silico* SNPs and indels. The web resource also houses various pathways where these genes are known to be involved. It has been developed using "*three tier architecture*," *namely,* a client tier, middle tier, and database tier (**Figure 1**). Client-end assists to display the information related to services available and build the front-end layer of the application for end-users of their customized search related to mastitis in buffalo. The database layer has been developed using PHP (Hypertext Preprocessor) which is an open-source server-side scripting language. The application layer, also known as the middle tier or logic tier takes the information from the presentation tier and controls the application's core functionality. Database tier has the information related to mastitis associated genes, gene pathways, proteins, and PPI, functions, and variants, etc. cataloged in MySQL database using SQL query language. *WBMSTDb* has been developed using LAMP (Linux-Apache-MySQL-PHP) technology and launched at Apache server.

RESULTS

Mining and Mapping of Bovine Mastitis Associated Genes

A total of 159 mastitis associated genes were sourced from literature, taking water buffalo (*Bubalus bubalis*) and cattle (*Bos taurus*) as reference. After removing the duplicates and alternative gene alias, 129 genes associated with mastitis were used for further study. Out of 129 unique genes associated with mastitis, 120 genes were retrieved from highly diverse cattle breeds like Canadian Holsteins, Holstein, Holstein Friesian, and Brown Swiss, Chinese Holstein, Sanhe cattle, Chinese Simmental, Luxi Yellow, and Bohai Black. The remaining 9 genes were reported both in cattle breeds mentioned and in buffalo breeds like Murrah and Egyptian buffalo.

A total number of 34,139 genes were retrieved from *Bubalus bubalis* from NCBI (30) for mapping. After manual curation, 34,067 genes (98.41%) were obtained, which were further used for mapping 129 mastitis associated genes. Of these, 101 genes were successfully mapped. **Supplementary Table 1** shows details including the mapping location (chromosome coordinates), chromosome number, strand orientation, the total number of exons, gene type, organism based on the literature survey of these genes, whether the information was retrieved from cattle or buffalo studies along with references. Chromosome-wise mapping of all these 101 genes is depicted in **Figure 2**.

Validation of Predicted TGP Genes in Water Buffalo Gene Expression Atlas

The mastitis associated genes under study are revalidated by using recently published work of the gene expression atlas (30) of the domestic water buffalo (*Bubalus bubalis*) containing 23,492 genes expressed across 50 different tissues. After removing the duplicates, the remaining 23,489 genes were used for the revalidation of 101 key candidate genes. It was observed that 85 out of 101 key candidate genes were common in 23,489 genes (**Figure 3**), except for genes that include: *selectin P (SELP), Amyloid beta precursor protein (APP), BTG anti-proliferation factor 1 (BTG1), lipopolysaccharide binding protein (LBP), CD46 molecule (CD46), lactotransferrin (LF), carboxypeptidase (CP), matrix metallopeptidase 9 (MMP9), BRCA1 DNA repair associated (BRCA1), RNA binding motif single stranded interacting protein 1 (RBMS1), ETS proto-oncogene 2, transcription factor (ETS2), transforming acidic coiled-coil containing protein 3 (TACC3), hepatocyte growth factor (HGF), serum amyloid A 3 (SAA3), orosomucoid 1 (ORM1)*, and *cytochrome c oxidase subunit II (COX2)*. The common gene list is included in **Supplementary Table 2**.

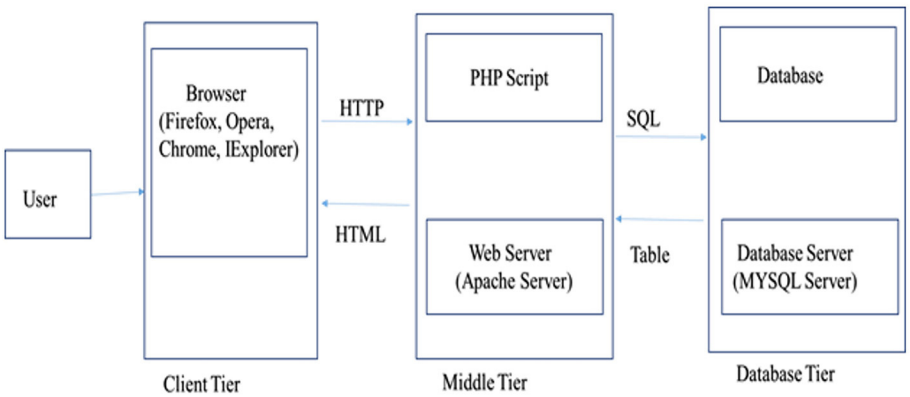

FIGURE 1 | Three tier architecture representing client, middle and database tier of *WBMSTDb*.

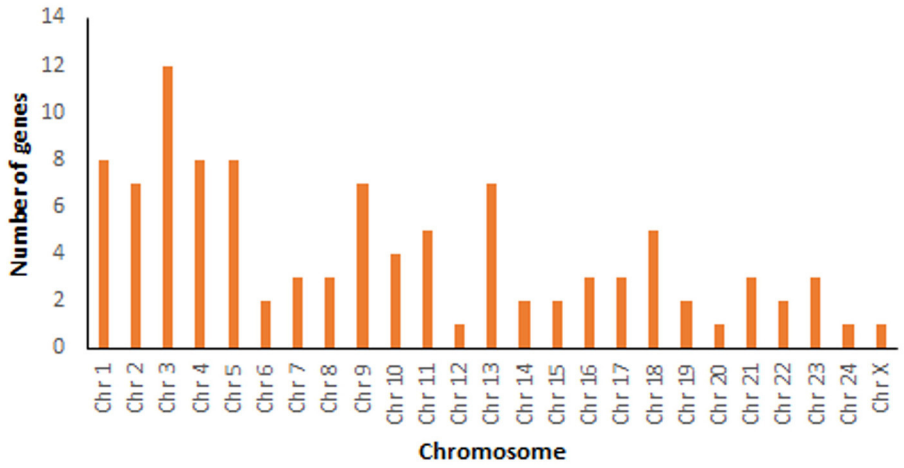

FIGURE 2 | Chromosome-wise mapping of 101 mastitis associated genes in *Bubalus bubalis*.

Functional Prediction of Mastitis Associated Candidate Genes

The roles of the mastitis associated genes in ontological terms were explored by their functional annotation. An organism specific classification system for the functionally annotated genes using PANTHER (32) was obtained for *Bos taurus* only as it does not have *Bubalus bubalis*. A genetically close bovine species, *Bos taurus* was used for this analysis to predict the biological role of the genes. Out of 101 genes, 98 were annotated successfully on molecular, biological, and cellular functions based on their role and classification to a PANTHER family. The predicted protein of mastitis associated genes and their pathways were also obtained. For example, gene *enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase (EHHADH)* are classified to PANTHER family, peroxisomal bifunctional enzyme (Family ID: PTHR23309). There are a total of 121 genes in this particular family. The molecular function of oxidoreductase activity and biological process includes fatty acid catabolic process and oxidation-reduction process. The protein class annotated for gene *EHHADH* is dehydrogenase. **Supplementary Table 3** includes details of all the 98 annotated genes in *Bos taurus*.

Gene annotation with species *Bubalus bubalis* using DAVID revealed annotation of 26 genes (**Table 1**). With respect to biological processes, genes such as *toll like receptor 2 and 4 (TLR2 and TLR4), C-C motif chemokine ligand 3 (CCL3), complement C3 (C3), C-X-C motif chemokine ligand 8 (CXCL8),* and *CD14 molecule (CD14)* were found to be involved in inflammatory response (44–47). These genes along with *S100 calcium binding protein A8 (S100A8)* and *lipopolysaccharide binding protein (LBP)* were involved in innate immune response (48, 49). *Interleukin1 beta (IL1B)* was involved in processes like fever generation, immune response, and positive regulation of cell division which provides evidence for the cause of symptoms in mastitis disease condition (50).

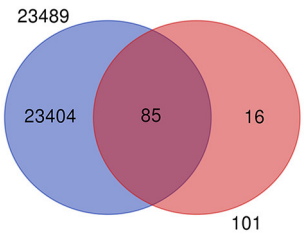

FIGURE 3 | Venn diagram showing the common and unique genes when compared to gene expression atlas of water buffalo and genes from milk traits.

The molecular functions in which most of these genes are involved are related to binding and kinase activity. For instance, *AKT serine/threonine kinase 3 (AKT3) and CCL3* are involved in kinase and ATP binding function (51, 52). *TLR2* and *TLR4* molecules are involved in transmembrane signaling receptor activity (53). Many genes are involved in calcium, metal, zinc, ion binding functions like *lactoperoxidase* (*LPO*), *calmodulin 2* (*CALM2*), *S100 calcium binding protein A8 (S100A8)*, in calcium-ion binding, *lactotransferrin (LTF)* in iron ion binding, and *tumor protein p53 (TP53)* in the metal-ion binding process (54–56).

Cellular processes include signal components, secreted molecules, glycoproteins, disulfide bond molecules, cytokines, and extracellular space. Maximum genes are involved in signaling processes, a list of genes is given in **Table 1**. *Interleukin 6 and 12B (IL6 and IL12B)* are involved in all the cellular processes as stated (57, 58). Protein family annotation for these 26 genes and information from protein databases such as Simple Modular Architecture Research Tool (SMART), Protein Information Resource (PIR), and InterPro is included with protein family ID and functions associated in *WBMSTDb*

TABLE 1 | Gene ontology analysis of candidate genes through DAVID.

Description	*P*-value	Genes	Fold enrichment	FDR
Cellular component				
Extracellular space	0.0002	*LTF, IL1B, LPO, CCL3, C3, IL12B, CXCL8, LBP, IL6*	4.22	0.12
Cytokine	0.0159	*IL1B, CCL3, IL12B, CXCL8, IL6*	4.46	14.48
Signal	0.0227	*IL6, CD14, LPO, CSF2, C3, IL12B, TLR4, TLR2, CCL3, LY96, CXCL8, LBP, IL6*	1.66	20.07
Glycoprotein	0.0905	*CCL3, CD14, LPO, IL12B, IL6*	2.68	60.35
Secreted	0.1675	*CD14, IL1B, LPO, LY96, IL12B, TLR4, CXCL8, IL6*	1.74	83.29
Disulfide bond	0.2239	*IL6, CCL3, CD14, LPO, CSF2, IL12B, CXCL8, IL6*	1.43	91.57
Biological process				
Immune response	0.3433	*IL1B, C3, CXCL8, IL6*	1.83	99.03
Inflammatory response	0.0095	*IL6, CCL3, LY96, IL12B, CXCL8, TLR2*	3.77	10.03
Innate immune response	0.0392	*IL6, S100AB, CCL3, TLR2, LBP*	3.35	35.68
Leucine-rich repeat	0.0439	*IL6, NOD2, CCL3, TLR2*	4.58	39.17
Inflammatory response	0.0567	*IL6, CCL3, IL1B, TLR2*	4.12	43.42
Immunity	0.0963	*IL6, CCL3, CD14, TLR2*	3.35	62.78
Innate immunity	0.2356	*IL6, CCL3, TLR2*	3.09	92.73
Membrane	0.9985	*IL6, CCL3, TLR2*	0.40	100.00
Molecular function				
ATP-binding	0.2356	*NOD2, AKT3, ACTB*	3.09	92.73
Nucleotide-binding	0.2913	*NOD2, AKT3, ACTB*	2.68	96.52
ATP binding	0.4803	*NOD2, AKT3, ACTB*	1.82	99.60

(59–61). The details are available at http://webtom.cabgrid.res.in/wbmstdb/index.php under the tab: "Search—Mastitis Gene Panel—Gene Annotation."

The pathway analyses of these genes as identified through KEGG Mapper revealed that out of the 101 mastitis associated genes under study, there were six genes for which KEGG ontology was not assigned. These are, *mannose-binding protein A (MBLA), actin beta (ACTB), FEZ family zinc finger 2 (FEZF2), osteoclast stimulating factor 1 (OSTF1), RNA binding motif single stranded interacting protein 1 (RBMS1), and RELA proto-oncogene, NF-kB subunit (RELA).*

A total of 225 pathways in various categories were classified, namely, metabolism, genetic and environmental information processing, cellular process, organismal systems such as immune system, and human diseases. The pathways revealed the involvement of these genes in diverse roles. The majority of these genes were involved in infection or disease susceptibility by microbial organisms like *Salmonella, Shigellosis, Pertussis, Legionellosis, Staphylococcus aureus, Yersinia,* etc. (**Table 2**).

Nine genes were found to be involved in apoptosis activity like *BCL2 associated X, apoptosis regulator (BAX), BCL2 related protein A1 (BCL2A1), MCL1 apoptosis regulator, BCL2 family member (MCL1), Fos proto-oncogene, AP-1 transcription factor subunit (FOS), growth arrest and DNA damage inducible beta (GADD45B), tumor protein p53 (TP53), AKT serine/threonine kinase 3 (AKT3), baculoviral IAP repeat containing 3 (BIRC3),* and *mitogen-activated protein kinase kinase 7 (MAP2K7).* **Table 2** describes the mastitis associated genes involved in various infectious pathways.

Prediction of Cross-Talking Proteins by Protein-Protein Network Analysis

The panels of 111 proteins were used for PPI network analysis. Among these, 10 proteins were buffalo specific, which were obtained directly from NCBI. The remaining 101 proteins were retrieved from the STRING database using their genetic codes. The construction of the PPI network revealed 83 nodes and 227 edges. These predicted proteins were further searched for other cross talking proteins in *Bubalus bubalis*. This resulted in a final PPI network of 193 nodes and 1,480 edges (**Table 3**). PPI predicted 1,707 proteins associated with mastitis disease in water buffalo. Based on the degree, between-ness, and closeness of the network, they were classified into two groups, *viz*., (1) directly interacting 83 proteins with cross-talk with 227 proteins as first-degree interaction (2) other predicted proteins (193) which are having cross-talks with 1,480 predicted proteins as second-degree interaction in the network.

Visualization of a network with highly connected nodes using Cytoscape detected the hub proteins (**Figure 4**). Hub molecule analysis revealed six protein nodes (>40 edges) that are highly connected, *namely*, *TP53* (number of edges: 56), *Signal transducer and activator of transcription 3 (STAT3)* (number of edges: 49), *IL1B* (number of edges: 46), *Jun oncogene (JUN)* (number of edges: 45), *AKT3* (number of edges: 41), and *acetyl-CoA acyltransferase 1 (ACAA1)* (number of edges: 40). Out of these, *TP53, STAT3, IL1B,* and *AKT3* were found to be the first interacting partners, i.e., key candidate genes involved in mastitis, while *JUN* and *ACAA1* were the other interacting partners. Furthermore, using Cytoscape plugins, *CentiScape,* and

TABLE 2 | Genes of mastitis involved in various infectious pathways.

Pathway	No. of genes	Genes involved
Helicobacter pylori infection	2	*CXCL8, CCL5*
Pathogenic *Escherichia coli* infection	7	*BAX, FOS, ILIB, TIRAP, IL6, CXCL8, TLR4*
Salmonella infection	12	*BAX, FOS, MAP2K7, AKT3, IL1B, LY96, TIRAP, IL6, CXCL8, TLR2, TLR4, BIRC3*
Shigellosis	11	*BAX, C3, TP53, AKT3, IL1B, CSF2, PLCE1, CXCL8, TLR4, CCL5, BCL2*
Yersinia infection	7	*FOS, MAP2K7, AKT3, IL1B, IL6, CXCL8, TLR4*
Pertussis	12	*CALM2, C4A, C3, FOS, IL1B, LY96, TIRAP, IL6, IL12B, IRF1, CXCL8, TLR4*
Legionellosis	9	*HSPA8, C3, IL1B, IL6, IL12B, CXCLS, TLR2, TLR4, BCL2*
Staphylococcus aureus infection	5	*CFB, C4A, C3, C5AR1, SELP*
Tuberculosis	12	*BAX, CALM2, C3, AKT3, IL1B, LBP, TIRAP, IL6, IL12B, TLR2, TLR4, NOD2*
Human T-cell leukemia virus 1 infection	7	*BAX, FOS, TP53, AKT3, IL6, CSF2, RELB*
Human immunodeficiency virus 1 infection	8	*BAX, CALM2, CCR5, FOS, MAP2K7, AKT3, TLR2, TLR4*
Measles	11	*BAX, HSPA8, FOS, TP53, AKT3, IL1B, STAT3, IL6, IL12B, TLR2, TLR4*
Influenza A	8	*BAX, AKT3, IL1B, IL6, IL12B, CXCL8, TLR4, CCL5*
Hepatitis B	11	*BAX, FOS, MAP2K7, TP53, AKT3, STAT3, TIRAP, IL6, CXCL8, TLR2, TLR4*
Hepatitis C	4	*BAX, TP53, AKT3, STAT3*
Herpes simplex virus 1 infection	10	*BAX, C3, TP53, AKT3, IL1B, IL6, IL12B, TLR2, CCL5, BIRC3*
Human cytomegalovirus infection	12	*BAX, CALM2, CCR5, TP53, AKT3, IL1B, STAT3, IL6, CCL3, CXCL8, PTGS2, CCL5*
Kaposi sarcoma-associated herpesvirus infection	14	*BAX, CALM2, C3, CCR5, FOS, MAP2K7, TP53, AKT3, STAT3, IL6, CSF2, CXCL8, PTGS2, FGF2*
Epstein-Barr virus infection	9	*BAX, GADD45B, MAP2K7, TP53, AKT3, STAT3, IL6, RELB, TLR2*
Human papillomavirus infection	6	*BAX, TP53, AKT3, SPP1, IRF1, PTGS2*
Amoebiasis	7	*IL1B, IL6, IL12B, CSF2, CXCL8, TLR2, TLR4*
Malaria	6	*IL1B, IL6, SELP, CXCL8, TLR2, TLR4*
Toxoplasmosis	10	*ALOX5, HSPA8, CCR5, AKT3, STAT3, LY96, IL12B, TLR2, TLR4, BIRC3*
Leishmaniasis	8	*C3, FOS, IL1B, IL12B, NCF4, TLR2, TLR4, PTGS2*
Chagas disease (American trypanosomiasis)	11	*C3, FOS, AKT3, IL1B, IL6, CCL3, IL12B, CXCL8, TLR2, TLR4, CCL5*
African trypanosomiasis	3	*IL1B, IL6, IL12B*

TABLE 3 | Network statistics of protein-protein interaction.

Network parameters	First interacting partners	Other interacting partners
Nodes	83	193
Edges	227	1480
Average node degree	5.47	15.3
Avg. local clustering coefficient	0.416	0.55
Expected number of edges	62	485
PPI enrichment *p*-value	<1.0e-16	<1.0e-16

MCODE, the highest PPI scores by three centrality methods were computed. These scores along with other parameters of these genes are shown in **Table 4**. A total of nine clusters identified from the PPI network were highly connected subnetworks. A cluster of highly connected proteins' sub-network revealed 22 nodes and 211 edges with a MCODE score value of 20 (**Figure 5**).

Evaluation of Mastitis TGP Based Variant Mining (SNPs and Indels)

The overall read mapping rate and concordant pair alignment rate of RNA-Seq data on the reference assembly was 94.4 and 88.9%, respectively. On mapping the genomic coordinates of SNPs and indels from the VCF file of mammary tissues transcriptome over coordinates of 101 genes using in-house Perl scripts, a total of 1,458 SNPs and indels were obtained. Out of these, 1,306 were SNPs and 152 were indels. The highest number of SNPs and indels (261) was observed for the gene, *BCL2* apoptosis regulator located on chromosome number 22 which is involved in apoptosis regulating activity. Gene *CD46* was mapped for 116 SNPs and indels. For the genes *MAF bZIP transcription factor F (MAFF), APP, signal sequence receptor subunit 1 (SSR1),* and *CP*: 42, 37, 36, and 36 SNPs and indels were mapped. Similarly, for 11 genes, *namely*, *uncoupling protein 3 (UCP3), DNA nucleotidylexotransferase (DNTT), C-C motif chemokine ligand 20 (CCL20), FEZ family zinc finger 2 (FEZF2), colony stimulating factor 2 (CSF2), CCL3, interleukin 6 (IL6), Acyl CoA thioesterase 1 (ACOT1), SAA3, MBLA*, and *Orosomucoid 1 (AGP)*, there was no SNP and indel mapping. **Supplementary Table 4** has the details of the SNP and indels mapped for the predicted mastitis genes.

Development of Web Resource: Water Buffalo-MSTdb (*WBMSTDb*)

Water Buffalo Mastitis Database (*WBMSTDb*) is an open source and user-friendly web resource of targeted gene panels,

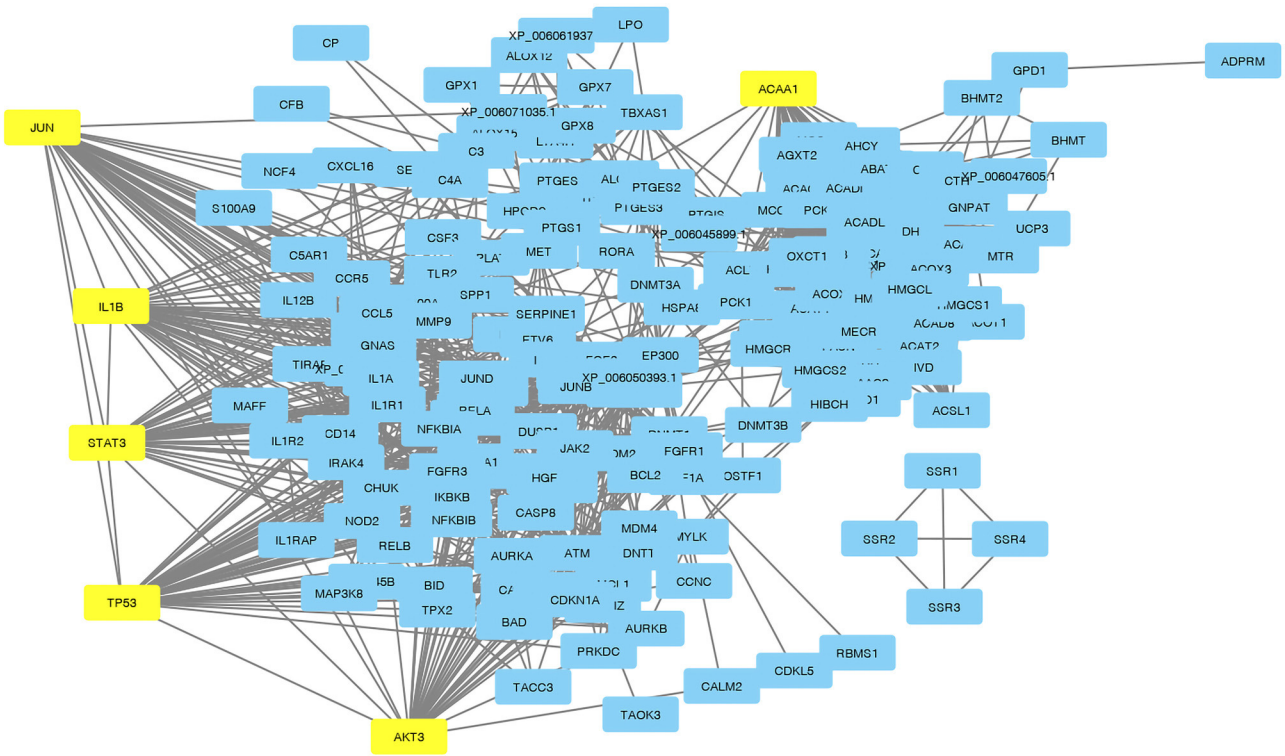

FIGURE 4 | Protein-protein interactions with hub genes highlighted in yellow.

which can be used for academic purposes in future mastitis association studies (website: http://webtom.cabgrid.res.in/wbmstdb/). WBMSTDb has five different tabs, *namely*, *Home, Search, Pathways, Tutorial,* and *Team*. *Home* tab deals with the brief introduction of this web resource along with various datasets related to mastitis cataloged in it. The objectives, developmental procedures, and rationale of its development are well-documented here. The *Search* tab is categorized into three sub-tabs, i.e., *Mastitis Gene Panel, Mastitis Disease Associated Proteins,* and *Mastitis Disease Associated Predicted Proteins*. Furthermore, the sub-tab *Mastitis Gene Panel* is categorized into two sub-sub-tabs, *namely*, "Gene Information" and "Gene Annotation." The "Gene Information" sub-sub-tab has the information related to Gene symbol, Gene ID, Gene Description, Chromosome Number, Gene Start Position, Gene End position, Orientation, and Exon Counts. Provision has been made for the browser for simpler visualization. As users may have targeted regions within the gene for SNP mining, like UTR region or exonic regions only, so to cater to such needs, provision has been made for the convenient visualization of regions of each ORF by integrating the TGP with the genome browser. The "Gene Annotation Information" sub-sub tab has information related to Gene ID, Gene name, Species, GO term BP (Biological processes), GO term CC (Cellular components), GO term MF (Molecular Functions), Interpro database, the PIR superfamily database, SMART database, and Up keywords. The "*Mastitis Disease Associated Proteins*" sub-tab provides information on Protein names, Length, and accession Numbers. The "*Mastitis Disease Associated Predicted Proteins*" sub-tab provides two types of interaction images, i.e., "*Major Interaction*" and "*Distant Interaction.*" The information of PPI provides Node1, Node2, Neighborhood chromosome, Gene fusion, Phylogenetic co-occurrence, Homology, Co-expression, Experimentally determined interaction, Database annotation, and Auto-text mining. The "*Function Annotation*" provides ProteinID, Description, Gene-observed, Gene-background, False-discovery rate, Matched proteinID, Database. The "*Pathways*" tab deals with the information like Pathways name, Pathways Id, Numbers of Enzyme, Enzyme, Sequence of Enzyme count, Sequence Id, and Pathway Figures. Users can download the targeted gene panel for very high coverage (600–1000X) sequencing to mine for extremely low frequency alleles potentially associated with mastitis disease. The *Tutorials* tab contains the guidelines for users and terminology used in the database contents. Finally, the *Team* tab has the contact list of researchers involved in the analyses and development of the web resource, *WBMSTDb*. The various interfaces for users are shown systematically in **Figure 6**.

DISCUSSION

Mining and Mapping of Bovine Mastitis Associated Genes

In a panel of successfully identified 129 mastitis associated genes, 110 and 19 genes were from cattle and buffalo populations, respectively. This availability of a higher number of mastitis genes

TABLE 4 | List of genes showing PPI scores >10 identified through MCODE and CentiScape.

Gene name	Score	Degree	Betweeness	Closeness
ACAA2	16.1739	38	94.5830	0.00231
ACAA1	16.1739	40	1590.0820	0.00257
ACADM	15.4545	37	173.4419	0.00230
MCCC1	15.3874	35	191.9848	0.00229
EHHADH	15.2138	39	700.0301	0.00246
HADHA	15.2138	36	117.0222	0.00223
ACAD8	15.2138	30	63.9670	0.00224
ACADL	15.1858	33	80.3648	0.00218
GCDH	15.1579	21	6.3206	0.00204
IVD	14.9674	30	42.3387	0.00219
PCCB	14.6147	36	421.9345	0.00234
HMGCL	14.5067	35	123.8824	0.00228
HMGCLL1	14.5067	35	123.8824	0.00228
ACOX3	14.4118	25	69.0563	0.00220
ACOX1	14.4118	38	2221.2560	0.00271
MCCC2	14.0870	32	76.7229	0.00226
HADHB	13.9355	39	109.0790	0.00234
HIBCH	13.5882	23	528.4855	0.00226
ACAT1	13.5665	34	60.6734	0.00228
ACAT2	13.5665	34	60.6734	0.00228
ACADS	13.4933	29	45.8871	0.00213
ACADVL	13.0850	29	65.4278	0.00224
ACADSB	12.7717	27	35.4102	0.00212
ABAT	12.4265	22	405.8688	0.00219
ACLY	12.0652	39	3099.4708	0.00279
ACSL1	12.0000	16	5.6126	0.00207
HIBADH	11.8105	25	71.9954	0.00201
HMGCR	11.7363	20	211.7744	0.00242
CS/Citrate Synthase Mitochondrial	11.6769	39	1462.1305	0.00240
OXCT1	11.5652	24	25.5766	0.00216
MECR	11.5429	16	50.0551	0.00208
HMGCS2	11.3116	28	392.3604	0.00247
AACS	11.1176	23	39.1651	0.00219

of cattle in GenBank is expected as cattle are a much more widely distributed and well-studied bovine species than water buffalo. The successful mapping of mastitis associated genes (101) of heterologous species cattle over the water buffalo genome reveals their extensive conservation during the divergence of these bovine species. This high conservation agrees with earlier mapping studies of water buffalo depicting syntenic block coverage of >80% (62). Many mastitis associated genes like *complement C5 (C5)* and *chemokine (C-X-C motif) receptor 1 (CXCR1)*, are reported to be conserved as high as up to 99% sequence similarity (63, 64).

This extensive conservation of cattle genes in water buffalo is due to their common ancestor, homologous proteins, common gene family, common CNV, common gene statistics, across species selection signature of milk pathways are also revealed in buffalo genome Benchmarking Universal Single-Copy Orthologs (BUSCO) mapping studies (62). The remaining 28 cattle genes, which could not be mapped over the buffalo genome, might be due to their acquired species specificity in cattle during species divergence (**Supplementary Table 5**). This structural genomic difference of river buffalo and cattle has also been reported in comparative genomic and transcriptomic studies (65).

The key candidate genes are mapped to the chromosome location and position using the entire set of buffalo genes from NCBI. Maximum key genes of mastitis were mapped to chromosome 3 as also reported in water buffalo previous studies (66, 67). Interestingly, many alleles of mastitis genes of chromosome 3 are also associated with fertility traits. Mastitis also affects fertility, as this disease may lead to early embryonic death, infertility, or sterility in water buffaloes (68).

Validation of Predicted TGP Genes in Water Buffalo Gene Expression Atlas

The successful mapping of 85% TGP genes over water buffalo species specific gene expression atlas demonstrates the reliability of predicted genes as they get transcribed in buffalo tissues. The remaining non-validated (15%) TGP genes might be due to spatiotemporal gene expression data in the atlas. In an atlas, all genes are not expected to express all the time. This will vary with the tissue as well as in the timeline for the sampling of transcriptome data generation. This spatiotemporal gene expression in the milk tissue of bovines has been reported (69).

Functional Prediction of Mastitis Associated Candidate Genes

Mastitis genes regulating the proteins may have a significant functional role in the modulation and regulation of mastitis disease. Proteins like *complement C4 (C4)*, *BRCA1, TLR4, MBLA*, and *calcium voltage-gated channel auxiliary subunit alpha2delta 1 (CACNA2D1)* are reported potential biomarkers in mastitis (70) which were also found in our study. The functional prediction of the key genes associated with mastitis disease was found to be involved in the immune and inflammatory response. For instance, genes like *CXCL8, CD14, S100A8, CSF2, C3, Interferon regulatory factor 1 (IRF1), IL1B, IL6, LPO, LBP, TLR2, and TLR4* are well-known immune and inflammatory responsive genes (71). Mastitis is a complex disease causing inflammation of the mammary gland where origin, severity, and outcome of the disease depend on the environment, pathogen virulence, and host immunity (9). Mastitis is characterized by physical, chemical, and biological changes in milk quality like the presence of blood, water, pus, clots, flakes, and shreds consisting of fibrin as well as cellular debris, udder enlargement, somatic cell count, and asymmetry of the gland (72). In our predictive functional annotation analysis, the key candidate genes were found to mediate such responses including mastitis fever generation (73).

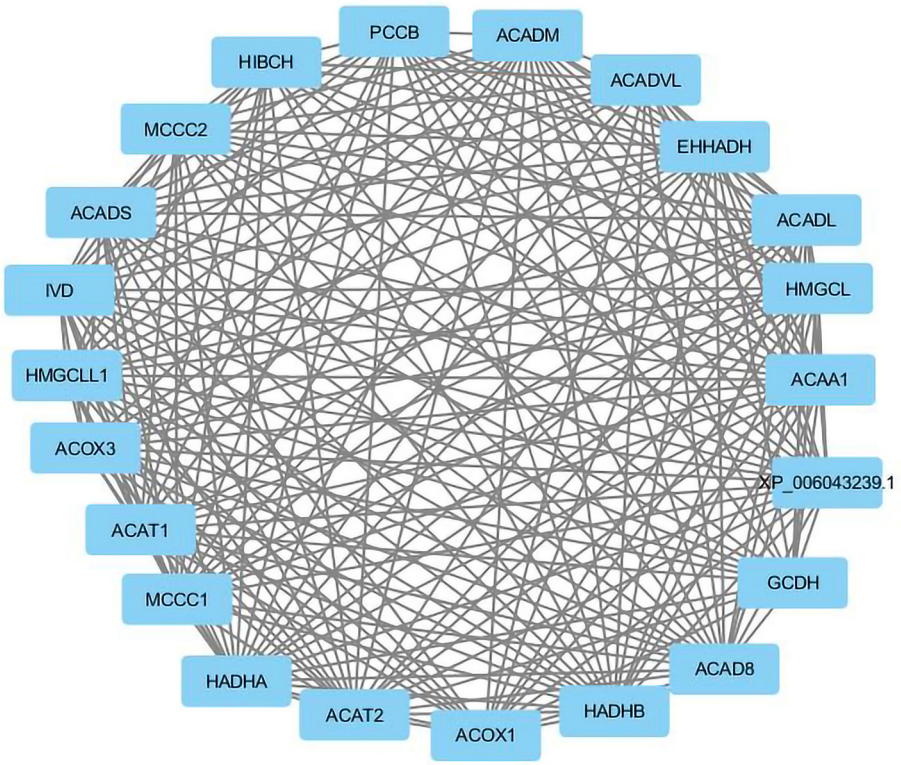

FIGURE 5 | The cluster of proteins highly connected in protein-protein interaction network.

Prediction of Cross-Talking Proteins by Protein-Protein Network Analysis

A protein-protein interaction study has revealed the various cross talking proteins of mastitis genes in water buffalo that can be used to understand the disease and associated proteins (74). Mastitis associated predicted proteins can be classified using the degree, between-ness, and closeness of the network (75). The top hub molecules of the interaction network predicted were *TP53, STAT3, IL1B,* and *AKT3*. The functional characterization of these proteins provides evidence of their role in signaling and the symptoms caused by mastitis. For example, *AKT3* expression increases during lactation in the mammary gland and immune cells, is also involved in the *TLR* pathways regulating as proinflammatory cytokines (51). The role of *IL1B* as an inflammatory cytokine in mastitis disease resistance has been reported in water buffalo (76). *TP53* gene has been reported as a key candidate gene that drives mastitis resistance genes (77). Furthermore, *IL1B* is reported as a hub molecule in cattle mastitis (78). The *Serotransferrin (TF)* gene is reported as a major regulator of a set of genes that are responsible for the resistance/susceptibility of mastitis (79). Enhanced *semaphorin 5A (SEMA5A)* gene expression has been reported to induce at least nine genes related to the host's immune response including *tumor necrosis factor alpha (TNF-a) and interleukin 8 (IL-8)* (80). Since the heritability of adaptive immune traits ranges from 0.25 to 0.35 in dairy cattle, thus the genes associated with it are valuable in mastitis resistance breeding (81). The high immune responder approach has been utilized to assess the dairy cattle's ability to trigger antibody and cell-mediated immune response against pathogens. The cows with superior adaptive immune response have been reported to show a lower occurrence of mastitis and metritis (82). How these predicted proteins have cross talk with mastitis associated proteins needs to be studied further to understand the genetic mechanism of this disease by a bottom up approach, predicting additional genes from these putative proteins. The genes of enlisted DAP can be used for future SNP discovery as well as for designing drugs (83).

Evaluation of Mastitis TGP Based Variant Mining (SNPs and Indels)

Variant mining for SNP (1,306) and indel (152) were found to be limited in number with respect to the total genes targeted (101). Since the genic region confines to <3% of the total genome, transcriptome data is expected to have less SNPs for WGS based SNP mining. In the case of water buffalo, the average exon length is 181.74 bp with respect to the average intron length of 3,435 bp, which also indicates a lower abundance of SNPs (62) in the genic region. This lower number of SNPs in genic region was also reported in water buffalo targeted sequencing in the Jafarabadi breed (84). In another study of the

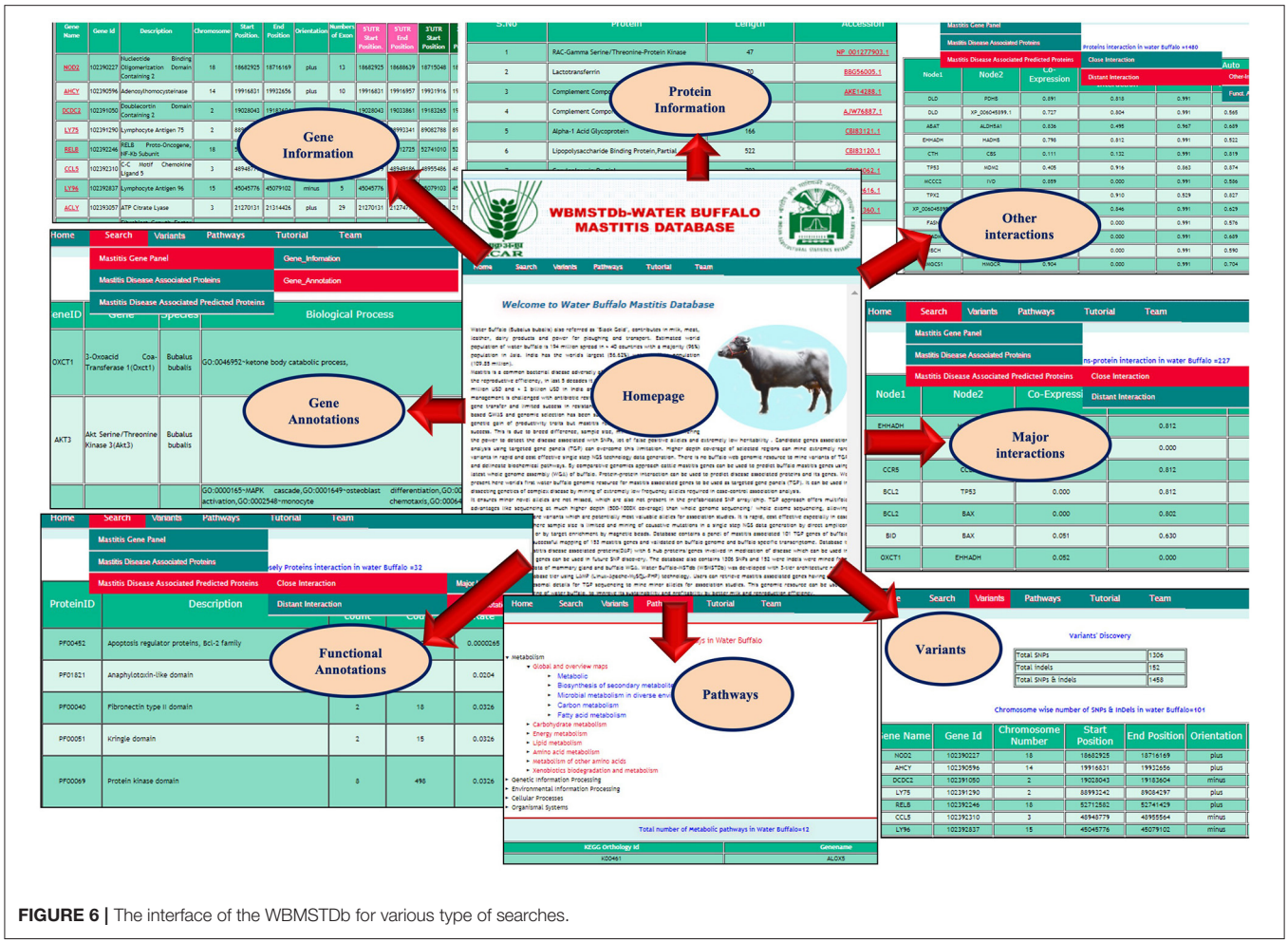

FIGURE 6 | The interface of the WBMSTDb for various type of searches.

Murrah water buffalo breed, genic region SNPs (79.40%) were found in nearly one-fifth of non-genic region SNPs (19.26%) in terms of their relative abundance (85). Though exonic variations are very limited in number, they do play an important role in population differentiation at the genetic level (86). Non-synonymous SNP polymorphism in the genic region of mastitis genes is very valuable for mastitis disease resistance breeding in bovines (87).

Such genic region SNPs with known physical map locations have been used in dissecting mastitis resistance QTL in dairy cattle (15). The use of indel as a marker has the advantage of making genotyping and multiplexing easier (88). Such indel polymorphism in milk genes and its trait association studies are reported in goats (89). A higher number of variants, i.e., 261 and 116, were found in gene *BCL2* (Gene ID 281020) and *CD46* (Gene 280851), respectively. A longer gene is expected to have a higher number of variants which is also evident by the higher number of exons (*BCL2* with 7 and CD46 with 17 exons). *BCL2* is also known to have copy number variants (90) and isforms (91), which might have contributed to the increased number of SNPs. The SNP of the *BCL2* gene has been found to be associated with heat stress in water buffalo (92). Similarly, the SNP of gene *CD46* was also found to be associated with mastitis in bovines (93). SNP studies of TGP genes can pave the knowledge discovery of pathway genes mediating mastitis disease by integrative analysis with transcriptomic data (24, 94).

Development of Web Resource: Water Buffalo-MSTdb (*WBMSTDb*)

The Water Buffalo-MSTdb (WBMSTDb) is the first targeted gene panel genomic resource of water buffalo for mastitis association studies. It is freely accessible to the global community. This web genomic resource can be used for the marker discovery required in mastitis resistance breeding of water buffalo. Users can download the complete ORF of the genes to be targeted for mining the variants. A genome browser can assist in the depiction of various parts of an ORF of the gene. Since TGP contains the entire open reading frame having 5′UTR, exons, introns, and 3′UTR with genomic coordinates, it can be used for SNP, simple-sequence repeat (SSR), and indel marker discovery, which are required in association studies for mastitis resistance breeding. Such marker discovery assay can be designed using NGS technology with the sequencing of very high depth coverage (500–1000X), by both target enrichment method (>50 gene

target genes) using magnetic beads or direct amplicon sequencing (if the number of genes are <50) in a single rapid cost-effective assay (25).

Many mastitis candidate gene association studies have been successfully reported for cattle. For example, in case of *mannan binding lectin serine peptidase 2 (MASP2)* (95), *TLR4* (96), *ATPase* Na^+/K^+ *transporting subunit alpha 1 (ATP1A1)* (97), *IL-8* (98), *mitogen-activated protein kinase kinase kinase kinase 4* (*MAP4K4*) (99), lipocalin-2 *(LNC2*) (100), *TLR2* (101), *CD14* (102), *TLR4* (103), Exon3 *TL-4* (104), *CD46* (93), *phosphodiesterase 9A (PDE9A)* (105), and *insulin-like growth factor 1 (IGF-1)* (106) have been reported. A similar approach is required in water buffalo for mastitis resistance breeding. *Target gene panel* approach with very limited sample size and limited gene number with case-control association study model has been successful in the diagnosis of susceptible individuals. A target gene panel covering both coding and non-coding region having SSR has been successfully used for improved diagnostic of disease (107).

CONCLUSION

The mapping of cattle mastitis associated genes in water buffalo genome assembly has revealed their extensive conservation. Mapping can be used advantageously in making targeted gene panels (TGP), which are required for low frequency variant mining in the water buffalo population. Such low frequency alleles are very valuable in disease association analysis and often get missed in GWAS. The limitations of GWAS studies in mastitis disease resistance breeding could be overcome by supplementing candidate gene association analysis approaches using our developed TGP in *WBMSTDb*. Protein-protein interaction has predicted disease associated protein and revealed the biochemical pathway mediating that takes place as a result of mastitis disease. These proteins can be used for future drug design. The predicted TGP genes were successfully validated in a transcriptome of various buffalo tissues. Successful variant mining was also done using the transcriptome data of the buffalo mammary gland. This TGP can be used in buffalo breeding programs for selection and culling and better milk and reproductive efficiency.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the web resource: http://webtom.cabgrid.res.in/wbmstdb/.

AUTHOR CONTRIBUTIONS

DK conceived the theme of the study. SJ, JJ, MI, JK, AG, and UA performed the computational analysis and developed genomic resources. SJ, JJ, MI, and DK drafted the manuscript. VN, SK, RK, TD, AR, and DK edited the manuscript. All co-authors read and approved the final manuscript.

ACKNOWLEDGMENTS

Authors are thankful to the Indian Council of Agricultural Research, Ministry of Agriculture and Farmers' Welfare, Government of India for providing financial assistance in the form of a CABin grant as well as the use of the Advanced Super Computing Hub for Omics Knowledge in Agriculture (ASHOKA) facility at ICAR-IASRI, New Delhi, India created under National Agricultural Innovation Project, and funded by the World Bank. All the co-authors wish to dedicate this manuscript and work to the COVID-19 warriors of India and the globe. We are extremely thankful to them because they make it possible to work safely and remotely. We also thank Dr. S. C. Gupta for critical suggestions and help, and Dr. Neelam Gupta, Animal Science Division, ICAR Head Quarter, New Delhi.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.593871/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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