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Potential activity of *Pleurotus nebrodensis* mushroom extract against biofilm of meticillin-resistant *Staphylococcus aureus and Pseudomonas aeruginosa*

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Mushroom extracts are a promising resource for treating bacterial infections associated with biofilms, offering a dual effect: inhibiting biofilm formation and facilitating infection healing. In this paper, after collecting in the wild basidiomes of *P. nebrodensis*, a rare and endangered species, we proceeded with the isolation of mycelium in pure culture. Preparation of the culture bags was entrusted to a leading fungiculture company. Cultivation took place in a purpose-built tunnel inside a farm, and after harvesting, a certain amount of mushrooms was dried and reduced to powder to proceed with the evaluation of antimicrobial activity against the multidrug-resistant bacterial strains *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*. *P. nebrodensis* extract showed reduction of biofilm formation by up to 71% for *S. aureus* and by 18% for *Ps. aeruginosa* compared to the untreated control. The results suggest that while the *P. nebrodensis* cold water extract does not exert direct antibacterial activity, it may interfere with biofilm formation, particularly in *S. aureus*, highlighting its potential as an anti-virulence agent. Besides, in both *Ps. aeruginosa* and *S. aureus*, treatment with the *P. nebrodensis* extract resulted in a reduction in biofilm formation compared to the untreated control. The potential of *P. nebrodensis* extracts in inhibiting biofilm formation processes by bacteria could lead to the discovery of new chemicals for use in clinical settings.

KEYWORDS

MDR bacteria, basidiomycota, biological activity, gram-positive bacteria, gram-negative bacteria, medicinal mushrooms, antibiotic resistance

1 Introduction

Pleurotus nebrodensis (Inzenga) Quèl. [Pleurotaceae, Basidiomycetes] is a rare, endemic, and declining mushroom from Sicily (Gargano et al., 2011). Molecular investigation on the *Pleurotus eryngii* species complex permitted clarification of the taxonomy of such species, which resulted clearly separated from the other *Pleurotus* Mediterranean taxa (Zervakis et al., 2014). Besides, commercial strains currently available in the international market and mislabeled under the name of "*P. nebrodensis*" in China were identified as *P. eryngii* subsp. *tuoliensis* (Venturella et al., 2016). *P. nebrodensis* is characterized by good nutritional content, and its organoleptic characteristics are appreciated by consumers (La Guardia et al., 2005). Preliminary studies demonstrated that *P. nebrodensis* possess *in vitro* antitumor (Fontana et al., 2014) and antibacterial activities (Schillaci et al., 2013) and can be included among the so-named "medicinal mushrooms". In the frame of a project funded by the Sicilian Regional Administration (Italy) and involving the cultivation of *P. nebrodensis* for food, medicinal, and phytoiatric purposes, investigation on the potential activity of the *P. nebrodensis* mushroom extracts against biofilm of methicillin-resistant Gram-positive and Gram-negative bacteria has been carried out. Numerous studies have highlighted that mushroom extracts represent a significantly more effective treatment against Gram-positive and Gram-negative bacteria compared to traditional antibiotics (Gargano et al., 2020). The global rise of antibiotic-resistant pathogens has emerged as a critical threat to public health, driven by the overuse and misuse of conventional antibiotics, as well as the rapid genetic adaptation of microbial populations. This alarming trend underscores the urgent need to explore alternative strategies, among which the bioprospecting of bioactive compounds from natural and organic resources—such as plants, marine organisms, and microorganisms—represents a promising and sustainable approach for identifying novel molecules with potent antimicrobial and therapeutic properties. The rapid emergence, selection, and dissemination of antibiotic-resistant bacteria therefore highlight the pressing necessity to develop innovative therapeutic strategies against infections caused by multidrug-resistant (MDR) microorganisms. Among these, the identification and application of natural compounds capable of enhancing the effectiveness of conventional antibiotics are gaining increasing attention. In particular, recent studies indicate that combining natural substances derived from fungi with commonly used antibiotics could represent an innovative and potentially transformative approach to overcoming resistance and effectively treating infections caused by MDR bacteria (Fasciana et al., 2021).

In this paper, the potential antimicrobial activity of *Pleurotus nebrodensis* was evaluated against clinical multidrug-resistant (MDR) strains of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*. These pathogens are not only among the most common causes of healthcare-associated infections, but are also characterized by their remarkable ability to produce biofilms and develop resistance to multiple classes of antibiotics. In particular, *Ps. aeruginosa* is an ubiquitous and opportunistic Gram-negative bacterium, known for its intrinsic resistance mechanisms and robust biofilm production, especially in immunocompromised patients. *K. pneumoniae*, another Gram-negative pathogen, is responsible for severe infections such as bacteremia and urinary tract infections, often exhibiting resistance to carbapenems and forming persistent biofilms. *E. coli*, while typically a harmless commensal of the human gut, includes pathogenic strains capable of acquiring virulence factors and antibiotic resistance, contributing to serious extraintestinal infections. *S. aureus*, a Gram-positive bacterium, stands out as one of the most adaptable human pathogens, notorious for its capacity to cause a broad spectrum of infections and for forming biofilms that enhance its resistance to both host defenses and antibiotic treatments (Schillaci et al., 2013; Gashaw et al., 2020).

2 Materials and methods

2.1 Sample collection, habitat details, and evaluation of morphological characters

Research carried out in pastures of Madonie Mts. (N. Sicily, Italy) led to the collection, in spring 2025, of *P. nebrodensis* sporophores growing as saprotrophs on the dead roots of *Prangos ferulacea* (L.) Lindl. (Apiaceae). Sporophores collected in Piano Battaglia (Madonie Mts), 37°52'39 "N-14°02'06 "E, 1687 m a.s.l. were used to obtain strains in the laboratory. The specimens were transferred to the Department of Agricultural, Food, and Forest Sciences (SAAF) of the University of Palermo, and stored at <4 °C for up to 24 h prior to morphological examination under a Leica MS5 binocular microscope, which was carried out according to Venturella and colleagues (Venturella et al., 2015). The nomenclature of vascular plants follow POWO (Plants of the World Online) while the nomenclature of fungi is referred to Index Fungorum.

2.2 Establishment of pure cultures and preparation of cultivation bags

The collected sporophores were dried and then deposited in the Herbarium of the Department of Agricultural, Food, and Forest Sciences (SAF 157). Prior to this, a piece of pseudo-tissue was removed from a fresh specimen, placed on potato dextrose agar (PDA) in Petri dishes under aseptic conditions, and incubated for 15 days at 25 ± 2 °C. The established pure culture (labelled as "strain 3a") was stored in the Mycotheca of the Herbarium SAF (SAF 220), and it was subsequently provided to the company Italmiko located in Senise (Potenza, Italy) for the preparation of spawn and the inoculation of mushroom substrates composed of weath straw and residues of sugar beets moistened and mixed together by a special machine. Then, the substrate was transferred to a mixing and dosing apparatus and packaged in heat-resistant bags weighing 4 kg. Sterilization was performed in autoclave, twice at 120 °C, 1.1 atm for 20 min with a 24 h interval between each cycle. After the

final cooling, substrates were inoculated using actively growing mycelium and incubation was carried out at 25 °C in the dark.

2.3 Tunnel of cultivation

The cultivation of *P. nebrodensis* (strain 3a), was carried out in a farm located in Gangi (Madonie Mts, northern Sicily) at altitude of ca. 1000 m: The greenhouse tunnel-shaped, has size of 26–30 m (length) x 9 m (width) x 3.0-3.5 m (height), and a total area of 234–270 m^2. The skeleton of the tunnel consists of galvanized steel beams that are placed at distances of 3 m from each other. The cover of the tunnel consists of a "sandwich" of materials arranged in the following way (from outside to inside): a) a sheet of transparent polyethylene (PE) with a thickness of 200 µm, b) a cotton wool insulation mattress (such as the material used to insulate walls or roofs of houses) with a thickness of 5–10 cm, and c) a sheet of PE on the inside. The floor of the tunnel is made of "rough concrete," which is a 10–15 cm layer of concrete reinforced with a galvanized steel mesh to facilitate washing and disinfection of the surfaces The tunnel is equipped with an air conditioning unit and a water mist generator and several sensors to detect the levels of climatic parameters (temperature, CO_2, relative humidity). The temperature inside the tunnel is 12 °C (or 15 °C during summer), ventilation is provided by fans of adequate power to support up to 10–15 complete exchanges of internal air volume per hour, humidity is about 98% while lighting is provided by artificial light.

2.4 Preparation of *P. nebrodensis* mushrooms powder

1 kg of basidiomes collected in the cultivation tunnels were thinly sliced and dried in a patented mushroom-drying appliance (Valla dryer, Borgotaro, Emilia Romagna, northern Italy) equipped with baskets for 24 hours. Next, the dried mushroom slices were placed inside a Bimby TM7 Multifunction Kitchen Robot in order to obtain a fine powder for analysis of antibacterial activity. The powder yield is 10% of the weight of the fresh mushroom. Figure 1 shows a flowchart summarizing the main methods used.

2.5 Bacterial strains enclosed in the study

The clinical strains of *S. aureus*, *Ps. aeruginosa*, *E. coli*, and *K. pneumoniae* examined were isolated from blood samples of patients with invasive infection. Bacterial identification was carried out using the BD Phoenix™ system (Becton Dickinson Europe Holdings SAS, Pont-de-Claix, France).

2.6 Activity against biofilm production

The minimal inhibitory concentration (MIC) of the *P. nebrodensis* mushroom extract was determined using a microdilution method, with serial dilutions of the extract (Gargano et al., 2025). The activity of the lyophilized powder derived from the mushroom was evaluated against four MDR clinical strains one Gram-positive and three Gram-negative bacteria respectively: *S. aureus*, *Ps. aeruginosa*. *E. coli* and *K. pneumoniae*. As a growth medium, either the tryptic soybean broth (Sigma-Aldrich, Darmstadt, Germany) containing glucose (2% w/v) or Mueller Hinton (Sigma-Aldrich, Darmstadt, Germany) was used. Bacterial strains were inoculated in TrypticaseTM Soy Agar (BBL TM; Becton, Dickinson and Company, Sparks, MD, USA) and incubated at 37 °C for 24 hours. In total, 100 µL of overnight cultures (0,5 McFarland in tryptose broth, BT) were added to a 96-well flat-bottom sterile plate (Biosigma S.r.l. Dominique Dutscher Group, Brumath, France). The negative control was composed of only half BT; to demonstrate biofilm production, excess bacterial suspension was removed. Subsequently, the sporophore powder extract (25% v/v) was re-suspended in BT, 100 µL were added to the wells and the cultures were incubated at 37 °C for 48 hours. Finally, the biofilm was stained with crystal

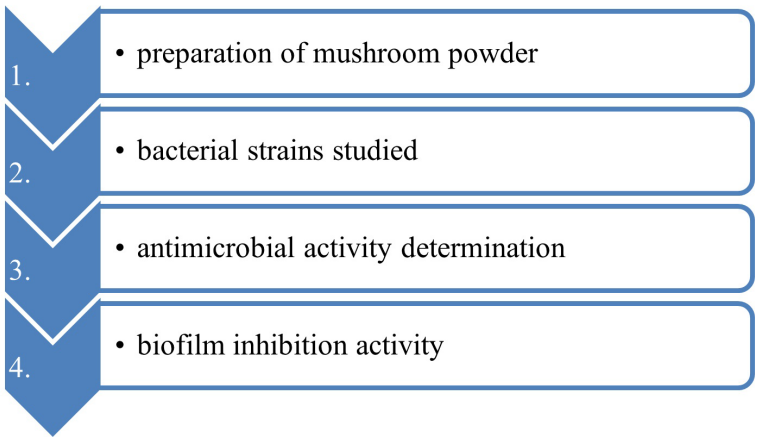

FIGURE 1
Flowchart of the methods used.

violet dissolved in ethanol (0.5% w/v). The optical density was measured at 570 nm (Spectrophotometer Multiskan Go; Thermo Fisher Scientific, Waltham, MA, USA). The experiment was conducted in triplicate (Calà et al., 2015). The effect of the extract obtained from the mushroom powder was also used to evaluate the ability to inhibit the biofilm production by *S. aureus*, *Ps. aeruginosa*, *E. coli* and *K. pneumoniae*.

3 Results

3.1 Morphological description of the *P. nebrodensis* specimen and development of mycelium

The basidiome of *P. nebrodensis* (Figure 2A) have a pileus 6.5 cm width, 10 cm length, ovoid or conch-shaped, creamy-white, fissured, with creamy-ochre areolae. The margin of the pileus is curved, entire and the flesh is compact, elastic, and white. The gills are decurrent, close together, light ivory, margin smooth., The stipe is smooth, cylindrical, light ivory, fibrous and stout, laterally attached to pileus. The microscopic features were evaluated with a Leica DLMB microscope using tap water. In particular we observed basidiospores 12.2-17.4 × 5.5-8.2 µm, cylindric-elliptic, smooth, hyaline, with drops (Figure 2B). Mycelium growth was quite fast in the pure cultures established, and the surface of the Petri dish (diameter 90 mm) was completely overgrown in 15 days (Figure 2C). The colony was found to be slightly floccose and flat, with dense white hyphae without any zonation and with a filiform margin. The inoculum (Figure 2D) was prepared by transferring a piece of pure culture into jars containing sterile soaked wheat grains, which were then incubated at 25°C until complete colonization.

3.2 Cultivation of *P. nebrodensis* strain 3a

In the year 2024, 1.000 bags inoculated with the *P. nebrodensis* strain 3a were placed in the tunnel of cultivation (Figure 3A). The mean yield per bag was 0.8 kg of mushrooms and the total yield of the tunnel was 800 kg of mushrooms. This value is near to the optimum value of production (1 kg of mushrooms per bag). The 70% of total yield (560 kg of mushrooms) was obtained with the first flush while the remaining 30% (240 kg of mushrooms) was produced during the second flush. The strain 3a has precocity at 16–18 °C (relative humidity 90%) and showed resistance to

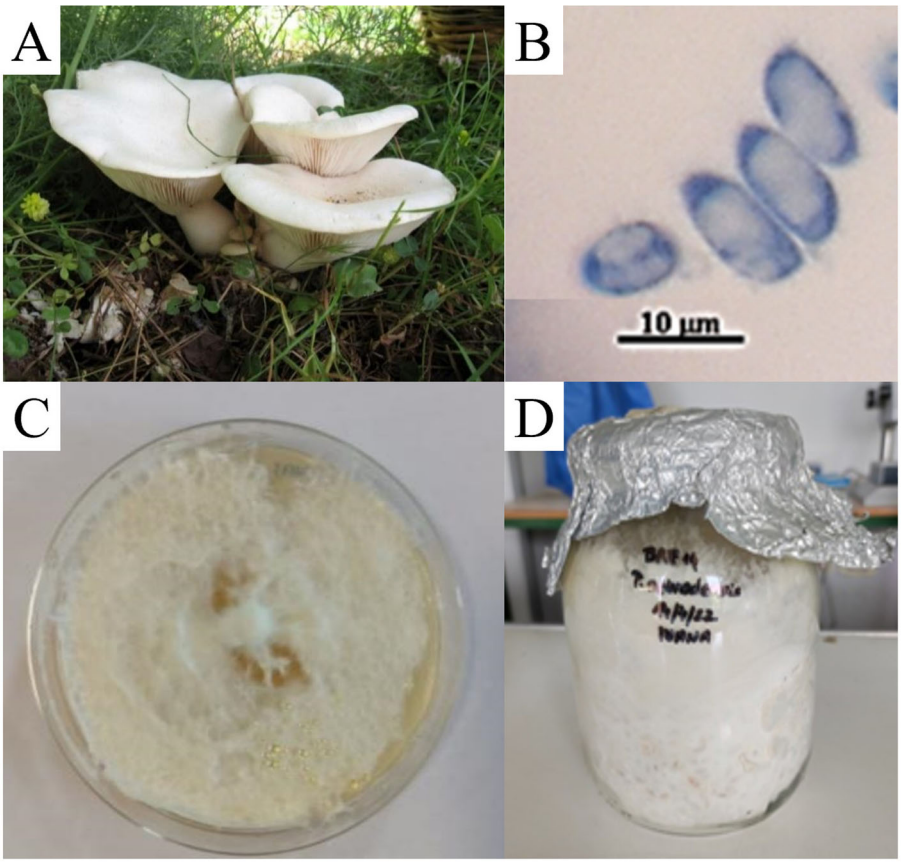

FIGURE 2
(A) Wild basidiomata of *P. nebrodensis*; **(B)** Basidiospores of *P. nebrodensis*. **(C)** Mycelium of *P. nebrodensis* (Strain 3a); **(D)** Wheat seeds inoculated with mycelium of *P. nebrodensis* (spawn).

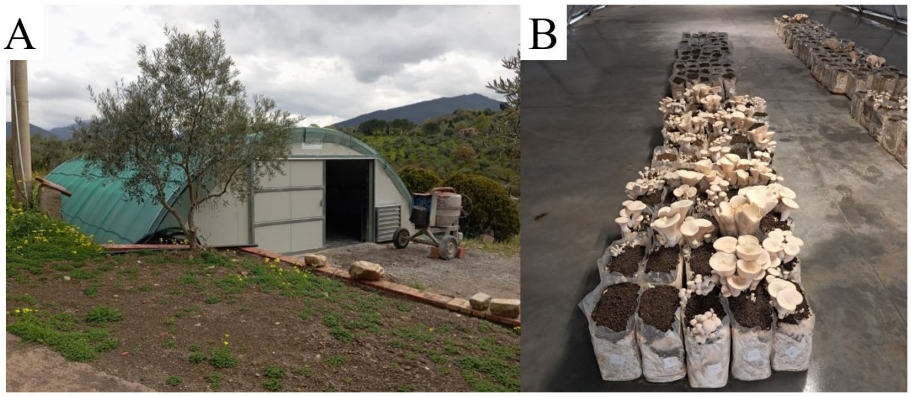

FIGURE 3
(A) Greenhouse tunnel-shaped for the cultivation of *P. nebrodensis*. **(B)** *P. nebrodensis* strain 3a, first flush.

bacteriosis which can occur with increases of temperature. The mushrooms are characterized by a beautiful white color, delicate smell and firm flesh, and reproduce the organoleptic characteristics of the wild mushroom (Figure 3B). They are much appreciated for their texture and delicate flavor.

3.3 *P. nebrodensis* mushroom powder

100 g of *P. nebrodensis* powder were obtained by 1 kg of cultivated fresh mushrooms (yield of 10%). The *P. nebrodensis* powder is thin, white and fragrant and was placed inside BRAND® PP graduated centrifuge tube, screw cap volume 50 mL, without base, sterile, to proceed with analysis of antibacterial properties.

3.4 Activity against biofilm production

Antibacterial activity assays revealed that the cold-water extract of *P. nebrodensis* did not exhibit inhibitory effects against the tested multidrug-resistant bacterial strains—*Ps. aeruginosa*, *S. aureus*, *E. coli*, and *K. pneumoniae*—even at the highest tested concentration of 50% v/v.

These results suggest that while the *P. nebrodensis* cold water extract does not exert direct antibacterial activity, it may interfere with biofilm formation, particularly in *S. aureus*, highlighting its potential as an anti-virulence agent. The results are shown in Figure 4.

The bar graph illustrates the effect of the *P. nebrodensis* cold water extract on biofilm formation by *Ps. aeruginosa* and *S. aureus*. Two conditions are compared for each bacterial species: untreated control and treatment with the *P. nebrodensis* extract (T+PN). In both *Ps. aeruginosa* and *S. aureus*, treatment with the *P. nebrodensis* extract resulted in a reduction in biofilm formation compared to the untreated control. The decrease in biofilm biomass is more pronounced in *S. aureus*, where the T+PN group shows a marked reduction relative to the NT group. No statistically significant effects were observed in *E. coli* and *K. pneumoniae* following treatment with the *P. nebrodensis* cold water extract, indicating a limited or absent

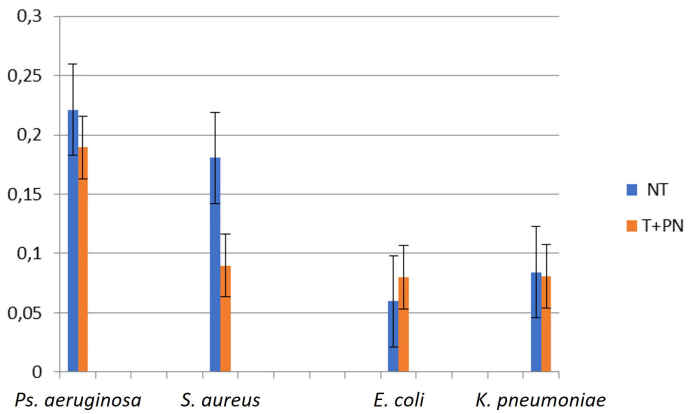

FIGURE 4
Effect of the *P. nebrodensis* cold water extract on biofilm formation by *Ps. aeruginosa*, *S. aureus*, *E. coli* and *K. pneumoniae* PN, *Pleurotus nebrodensis*; NT, untreated control.

impact on biofilm formation in these strains under the tested conditions.

4 Discussion

The results obtained from the use of aqueous mushroom extracts, specifically from *P. nebrodensis*, demonstrate significant therapeutic potential in combating biofilm formation by pathogenic bacteria such as *S. aureus* and *Ps. aeruginosa*. These two bacterial species are well known for their role in causing persistent and difficult-to-treat infections, especially in immunocompromised patients, where standard antibiotic therapies often fail. Remarkably, the application of *P. nebrodensis* extracts has been shown to reduce biofilm formation by up to 71% in *S. aureus* and by approximately 18% in *Ps. aeruginosa*. Such a reduction is of considerable clinical importance because biofilms significantly contribute to bacterial virulence and chronic infection persistence (Hernández-Jiménez et al., 2022; Duhaniuc et al., 2024).

Biofilms, which are complex communities of bacteria embedded within a self-produced extracellular polymeric matrix, act as a protective barrier that shields bacteria from both host immune defenses and the effects of conventional antibiotics. This protective environment enables bacteria to survive in hostile conditions, leading to increased resistance to antimicrobial agents and complicating treatment outcomes (Rather et al., 2021). Within this context, the discovery of natural agents capable of disrupting or preventing biofilm formation represents a critical advancement in antimicrobial therapy. The anti-biofilm action of mushroom extracts, such as those from *P. nebrodensis*, offers an innovative and complementary therapeutic strategy. By inhibiting the initial adhesion and subsequent maturation of biofilms, these natural compounds enhance the susceptibility of bacteria to antibiotics and immune system clearance mechanisms (Garcia et al., 2022).

Furthermore, the integration of natural substances like *P. nebrodensis* extracts into treatment regimens may play a pivotal role in reducing the overuse and misuse of antibiotics, which is a major contributing factor to the global escalation of antimicrobial resistance. This is particularly significant as antibiotic resistance poses a severe threat to public health worldwide, leading to increased morbidity, mortality, and healthcare costs. The use of mushroom extracts could thus serve as a valuable alternative or adjunct therapy, potentially restoring antibiotic efficacy and extending their clinical utility. Combining these extracts with conventional antibiotics might synergistically improve therapeutic outcomes by weakening biofilm defenses and allowing better drug penetration (Alves et al., 2014).

In summary, mushroom extracts, especially those obtained from *P. nebrodensis*, emerge as a promising resource for addressing bacterial infections associated with biofilm formation. They offer a dual beneficial effect: not only inhibiting the establishment and growth of biofilms but also facilitating the resolution of infections through enhanced immune system engagement and antibiotic action. The bioactive compounds within *P. nebrodensis* extracts have the potential to be developed into novel antimicrobial agents or adjuvants, thereby contributing to the advancement of clinical treatments for biofilm-associated infections. However, to fully harness this potential, further detailed research is necessary to isolate and characterize the specific active ingredients responsible for the observed antibiofilm effects. Additionally, understanding the molecular mechanisms underlying their mode of action will provide critical insights that could guide the design of more effective and sustainable therapeutic interventions. This line of investigation opens promising new avenues for the development of innovative strategies to combat antibiotic-resistant bacterial infections, ultimately improving patient outcomes and public health.

Data availability statement

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

Author contributions

MG: Project administration, Resources, Writing – original draft, Validation, Methodology, Conceptualization, Supervision, Funding acquisition, Investigation. IC: Investigation, Data curation, Writing – original draft, Visualization, Formal analysis. FC: Visualization, Data curation, Formal analysis, Investigation, Writing – original draft. AG: Conceptualization, Validation, Supervision, Methodology, Writing – review & editing. VT: Data curation, Writing – original draft, Visualization, Investigation. IA: Visualization, Formal analysis, Writing – original draft, Investigation. TF: Writing – original draft, Validation, Conceptualization, Writing – review & editing, Methodology, Supervision.

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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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