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## EDITED BY

Arabela Untea,  
National Research Development Institute for  
Animal Biology and Nutrition, Romania

## REVIEWED BY

Igor Popov,  
Don State Technical University, Russia  
MD. Shariful Islam,  
University of Rajshahi, Bangladesh

## \*CORRESPONDENCE

Elena Franco Robles  
✉ e.franco@ugto.mx

<sup>†</sup>These authors have contributed  
equally to this work and share  
first authorship

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# Bioactive-enriched feed containing agave fructans and turmeric powder as an alternative to antibiotics in early-laying hens

Cristina D. Balcón Pacheco<sup>1†</sup>, Carlos A. García Munguía<sup>2†</sup>,  
César Ozuna<sup>3</sup>, Román Cardona Herrera<sup>1</sup>,  
Alberto M. García Munguía<sup>4</sup> and Elena Franco Robles<sup>2\*</sup>

<sup>1</sup>Division of Life Sciences, University of Guanajuato, Irapuato, Mexico, <sup>2</sup>Department of Veterinary and Animal Science, Division of Life Sciences, University of Guanajuato, Irapuato, Mexico, <sup>3</sup>Department of Food Science, Division of Life Sciences, University of Guanajuato, Irapuato, Mexico, <sup>4</sup>Department of Agronomic Sciences, Center for Agricultural Sciences, Autonomous University of Aguascalientes, Aguascalientes, Mexico

The increasing restrictions on antibiotic use in poultry production have prompted the search for natural alternatives that improve health and productivity in laying hens. This study aimed to evaluate the effects of dietary supplementation with agave fructans (PRE), turmeric powder (AOX), and their combination (PA) on productive performance, egg quality, hematological status, and gastrointestinal traits in early-laying hens. A total of 120 Lohman White hens (20 weeks old) were randomly assigned to five dietary treatments: basal control (CON), antibiotics (ATB), agave fructans (PRE), turmeric powder (AOX), and the combination of both additives (PA). Results showed that agave fructans improved internal egg quality, increasing albumen and yolk height and egg weight without altering feed intake or conversion. Both PRE and AOX enhanced immune status, evidenced by a reduced heterophil-to-lymphocyte ratio and modulation of leukocyte populations. Gastrointestinal traits were minimally affected, with only slight variations in crop and cecal pH and duodenal organ weight. In conclusion, PRE supplementation provided the most consistent physiological benefits, supporting its use as a sustainable strategy to improve egg quality and welfare in laying hens while reducing reliance on antibiotics.

## KEYWORDS

bioactive-enriched feed, agave fructans, turmeric, keyword, egg quality, poultry nutrition, antibiotic alternatives, sustainable livestock production

# 1 Introduction

Over the last three decades, the demand for eggs has increased by 150%, surpassing the demand for meat and milk (FAO, 2023). Due to this, the production of laying hens has intensified, which has implications for bird welfare, including an increased risk of disease transmission. The use of antibiotics—either as growth promoters or for disease prevention and treatment—in large-scale production has led to the emergence of antimicrobial resistance. The selective pressure and horizontal transfer of antibiotic resistance genes among multiple bacterial species that cause human disease are of fundamental importance to the One Health concept and global health security, particularly in low- and middle-income countries (Kim and Cha, 2021; Cella et al., 2023). Furthermore, antibiotics used as feed additives have been detected as residues exceeding maximum residual limits in animal products, including eggs (Owusu-Doubreh et al., 2023). In this context, studies have shown that consumers are more concerned about chemical risks in food, such as antibiotic residues, than about microbiological risks (Meagher, 2019).

As an alternative to the use of antibiotics in poultry production, various compounds, mainly of natural origin, have been evaluated. The popularity of the use of feed additives in the feeding of production animals has increased in recent decades, especially in the production of animal-derived foods intended for consumers who prefer organic, cruelty-free, and safe foods. Accordingly, nutritional supplements such as prebiotics, probiotics, herbal extracts, and others have been investigated for their potential to enhance growth performance and health in livestock and poultry (Lao et al., 2020; Bąkowski and Kiczorowska, 2021; Oluwafemi et al., 2020; Al-Baadani et al., 2021) and are able to replace the use of antibiotics as a feed additive (Balcón-Pacheco et al., 2023; Franco-Robles and Hernández-Granados, 2024).

The inclusion of prebiotics such as fructans in the diet of laying hens has been shown to improve feed conversion, modify intestinal pH, increase calcium absorption and retention, and improve egg production and quality by reducing cholesterol content (H. M. Shang et al., 2010; Świątkiewicz et al., 2010; Gaggia et al., 2010). Fructans derived from *Agave tequilana* Weber var. Blue, known as agavins, are prebiotics characterized by a highly branched fructose structure with  $\beta(2-1)$  and  $\beta(2-6)$  glycosidic bonds and terminal glucose units (Lopez et al., 2003).

Turmeric powder from *Curcuma longa*, which contains high concentrations of polyphenolic compounds, has also been used in poultry feed, often in conjunction with other plant extracts, such as oregano and thyme, among others, and even using supplementation with vitamin E (Rajput et al., 2013). Studies have shown that turmeric and its derivatives improve laying percentage, feed conversion ratio, and egg quality parameters, including shell thickness, yolk and albumen index, Haugh units, and yolk pigmentation, while enhancing antioxidant content and reducing total cholesterol (Rajput et al., 2013; Kermanshahi and Riasi, 2006; Radwan Nadia et al., 2008; Riasi et al., 2012; Khan et al., 2012; Arshami et al., 2013).

Despite extensive research on prebiotics and phytogetic additives, few studies have examined the synergistic potential of agave fructans and turmeric powder as a combined natural alternative to antibiotics in laying hens. This study addresses this gap by evaluating their individual and combined effects on productive performance, physiological parameters, and gastrointestinal characteristics in early-laying hens.

# 2 Materials and methods

## 2.1 Ethics statement

The experiment was conducted at the Department of Veterinary and Animal Science, University of Guanajuato, Campus Irapuato–Salamanca, Division of Life Sciences, Mexico. All procedures carried out within the experimental protocol were approved by the Institutional Bioethics Committee of the University of Guanajuato (approval code: CIBIUG-P42-2019; approved on July 5, 2019).

## 2.2 Animals, management, and experimental design

A total of 120 healthy 18-week-old Lohman White hens were obtained from a commercial hatchery (Jalisco, Mexico) and housed individually in pens (25 cm x 35 cm x 40 cm; floor slope, 12°) under controlled environmental conditions (25–25°C) with 16 h of continuous light and 8 h of darkness per day. Feed and purified water were provided *ad libitum*. The hens underwent a 2-week adaptation period before the experimental phase.

At 20 weeks of age, hens were randomly assigned to five dietary treatments, with 24 replicates per diet, as follows: CON (basal diet without additives), ATB (antibiotics, basal diet + 0.02% colistin sulfate + 0.05% zinc bacitracin), PRE (basal diet + 0.5% agave fructans), AOX (basal diet + 0.5% turmeric powder), and PA (basal diet + 0.5% agave fructans + 0.5% turmeric powder). The hens were fed the experimental basal diet for 12 weeks (21–32 weeks of age). The composition of the basal diet, formulated according to the nutritional requirements outlined in the Lohmann Laying Hens Management Guide, is presented in Table 1. Colistin sulfate combined with zinc bacitracin was included in the ATB group to represent a conventional antibiotic program historically used in commercial poultry diets in Mexico, enabling comparison with functional feed additives while maintaining alignment with antimicrobial stewardship principles.

## 2.3 Composition of feed functional ingredients

The agave fructans used were a crystalline, water-soluble powder obtained from *Agave tequilana* Weber var. Azul,

TABLE 1 Ingredients and calculated nutrient analysis of standard diet.

| Ingredients (%)                         | Basal diet |
|-----------------------------------------|------------|
| Corn                                    | 44.30      |
| Soybean meal                            | 19.50      |
| Wheat bran                              | 17.50      |
| Cane molasses                           | 4.00       |
| Vegetable oil                           | 1.00       |
| Limestone                               | 10.00      |
| Orthophosphate                          | 1.50       |
| L-lysine HCl                            | 0.2        |
| L-methionine                            | 0.2        |
| L-threonine                             | 0.1        |
| Salt                                    | 0.3        |
| Vitamin and mineral premix <sup>a</sup> | 1.0        |
| Total                                   | 100.00     |
| Calculated provisions                   |            |
| Metabolizable energy (MJ/kg)            | 11.60      |
| Crude protein (%)                       | 18.70      |
| Crude fiber (%)                         | 3.11       |
| Ca (%)                                  | 4.00       |
| Available P (%)                         | 0.38       |

<sup>a</sup>The contribution of vitamin and mineral premix was supplemented at the rate of: vitamin 39,378 I.U./g, vitamin D3 5.358 I.U., vitamin B1 8.17 mg, B2 21.60 mg, B6 16.66 mg, B12–108 mcg, pantothenic acid 82.5 mg, folic acid 5.3 mg, copper 36 mg, biotin 7 mg, selenium 0.75 mg, zinc 360 mg.

containing 90.7% inulin, 5.1% fructose, 2.0% glucose, 1.8% sucrose, and 0.4% other carbohydrates, as determined by high-performance liquid chromatography (HPLC) with refractive index detection (Agilent 1260, Grupo Solalve, Jalisco, Mexico).

The turmeric powder was a commercial yellow–orange powder (>98% curcuminoids) purchased from a commercial supplier (Expo Alimentos, Jalisco, Mexico). To characterize its antioxidant properties, the total phenolic compounds, total flavonoids, carotenoids, chlorophyll content, and *in vitro* antioxidant capacity were evaluated as described below:

2.3.1 Total phenolic compounds

Extracts used for the determination of total phenolic compounds (TPC), total flavonoids (TF), and antioxidant capacity *in vitro* were prepared according to (Cardona-Herrera et al., 2025). The TPC was measured as described by (Cardona-Herrera et al., 2025). The reaction was incubated for 1 h in the dark, and absorbance was recorded at 765 nm using a UV–Vis spectrophotometer (Genesys 10S, Thermo Scientific, Waltham, USA). Results were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

2.3.2 Total flavonoid content

The total flavonoid content (TFC) was determined using the aluminum chloride method according to (Castañeda-Salazar et al., 2023). The reaction mixture was read at 415 nm using a UV–Vis spectrophotometer (Genesys 10S, Thermo Scientific, USA). Results were expressed as milligrams of quercetin equivalents per gram of dry weight (mg QE/g DW).

2.3.3 Carotenoids and chlorophyll content

Extracts used for determining carotenoid (CAR) and chlorophyll (CHLO) content were obtained following the procedure of (Samakradhamrongthai et al., 2021). A 2 mL aliquot of the extract was used to determine the CAR and CHLO content. The absorbance was measured with a UV–Vis spectrophotometer (Genesys 10S, Thermo Scientific, Waltham, USA). CAR<sub>R</sub>, and CAR<sub>Y</sub> (470 and 508 nm) and CHLO (646 and 663 nm), concentrations were determined according to Equations 1, 2, and 3 proposed by (Lichtenthaler, 1987; Hornero-Méndez and Mínguez-Mosquera, 2001). Results were expressed as micrograms per gram of dry weight (μg/g DW).

CHLO<sub>a+b</sub> = 7.15A<sub>663</sub> + 18.71A<sub>646</sub> (1)

CAR<sub>R</sub> (μg/mL) = (A<sub>508</sub> × 2144.0) − (A<sub>472</sub> × 403.3) / 270.9 (2)

CAR<sub>Y</sub> (μg/mL) = (A<sub>472</sub> × 1724.3) − (A<sub>508</sub> × 2450.1) / 270.9 (3)

2.3.4 Antioxidant capacity in vitro

The Trolox equivalent antioxidant capacity (TEAC) was determined following the method described by (Cardona-Herrera et al., 2025). First, 250 μL of extract was mixed with 2250 μL of 60 μM 2,2-diphenyl-1-picrylhydrazyl (DPPH), and absorbance was measured at 517 nm using a UV–Vis spectrophotometer (Genesys 10S, Thermo Scientific, Waltham, USA). Results were expressed as micromoles of Trolox equivalents per gram of dry weight (μmol TE/g DW).

2.4 Egg sample collection and measurements

Starting at 20 weeks of age, egg production (EP) was recorded daily until the hens reached 32 weeks of age. Body weight was measured weekly. Average daily feed intake (ADFI) was calculated by subtracting the leftover feed weight from the amount provided the previous day.

The egg mass (EM) and feed conversion ratio (FCR) were calculated according to Equations 4 and 5, while the relative organ weight (ROW) was determined as shown in Equation 6.

EM (g/hen) = EP × EW / 100 (4)

The feed conversion ratio (FCR) was calculated using (5):

$$FCR = \frac{ADFI(g)}{EP(g)} \quad (5)$$

## 2.5 Egg quality parameters

At three points during the experiment—T0 (week 21), T1 (week 27), and T2 (week 32)—a total of 90 eggs (six eggs per treatment at each time point) were randomly selected to determine egg quality traits. Egg length (EL, cm), egg width (EWD, cm), eggshell thickness (EST, mm), albumen height (AH, mm), thick albumen (TA, mm), thin albumen (tA, mm), yolk height (YH, mm), and yolk width (YW, mm) were measured using a Vernier digital caliper (HER-411, Steren, Mexico). Eggshell weight (ESW, g) was measured using an analytical balance (VE-204, Velab, Mexico). The egg quality index (EQI) was calculated using equations introduced by (Narushin et al., 2021). The color was assessed using the Yolk Color Fan (DSM, USA), while yolk color profile parameters—lightness ( $L^*$ ), redness ( $a^*$ ), and yellowness ( $b^*$ ) values were determined using a Hunterlab Colour Flex (Hunterlab, USA).

## 2.6 Sampling and hematological and biochemical parameters

At the end of the experiment, 10 birds were selected from each dietary treatment, and blood samples (3 mL) were collected from the wing vein into heparinized tubes (BD Vacutainer®) after a 12-h feed withdrawal. Plasma was obtained by centrifugation at 3000 g for 15 min at 4°C and stored at -20°C for further analysis.

Hematocrit was measured by the micro method using capillary tubes, centrifuged at 2500 g for 10 min. The counting of total leukocytes (TLC) was performed (Balcón-Pacheco et al., 2023). To calculate total erythrocyte count (TEC), the cells of the five quadrants were added and divided by 100, reporting the number of erythrocytes  $\times 10^6/\mu\text{L}$ . A peripheral blood film stained with Hemocrom-Fix (Golden Bell, Mexico) was performed for the differential count of heterophils, lymphocytes, monocytes, eosinophils, and basophils. Plasma glucose, calcium, and phosphorus levels were analyzed using commercial diagnostic kits (Spinreact®, Mexico).

## 2.7 Relative organ weight

The birds used for blood sampling were euthanized by cervical dislocation. The crop, proventriculus, gizzard, duodenum, jejunum, ileum, large intestine, and ceca were dissected and collected. The contents of each organ were used for pH determination using a potentiometer (Hanna Hi98103, Hanna, USA).

Relative organ weight (ROW) was calculated using the following equation:

$$ROW = \text{organ weight (g)} \div \text{body weight (g)} \quad (6)$$

The lengths of the small intestine, large intestine, and ceca were measured with a Vernier caliper and expressed in millimeters (mm).

## 2.8 Statistical analysis

Each replicate was treated as an experimental unit. Data normality was assessed using the Kolmogorov–Smirnov test. A one-way analysis of variance (ANOVA) was performed using Statistica (version 8.0; StatSoft, USA). When significant effects were observed, Bonferroni's *post hoc* test was used to adjust for multiple comparisons and to control the overall Type I error rate. A *p*-value of < 0.05 was considered statistically significant.

## 3 Results

Table 2 shows the total bioactive compounds and *in vitro* antioxidant capacity of the turmeric powder (TP) included in the laying hen diet. The total phenolic content (TPC) and total flavonoid content (TF) were  $4.76 \pm 0.15$  mg GAE/g DW and  $6.40 \pm 0.16$  mg QE/g DW, respectively, representing the main contributors to the antioxidant potential of the sample. Yellow carotenoids ( $\text{CAR}_Y$ ) were detected at  $3.41 \pm 0.23$  mg/g DW, whereas red carotenoids ( $\text{CAR}_R$ ) were not detected. Chlorophyll (CHLO) content reached  $30.96 \pm 0.91$   $\mu\text{g/g}$  DW. Antioxidant capacity assays revealed values of  $10.55 \pm 0.67$   $\mu\text{mol TE/g}$  DW for TEAC and  $58.74 \pm 0.62$   $\mu\text{mol TE/g}$  DW for FRAP, indicating a strong reducing capacity of the turmeric matrix. Overall, these results confirm that the TP used in this study contained considerable amounts of phenolic and flavonoid compounds associated with high antioxidant activity.

Table 3 summarizes the productive performance of laying hens fed diets containing different additives. No significant differences ( $p > 0.05$ ) were observed in body weight (BW),

TABLE 2 Total bioactive compounds and *in vitro* antioxidants of turmeric powder.

| Item                                 | Levels           |
|--------------------------------------|------------------|
| TPC (mg GAE/g DW)                    | $4.76 \pm 0.15$  |
| TF (mg QE/g DW)                      | $6.40 \pm 0.16$  |
| $\text{CAR}_Y$ (mg/g DW)             | $3.41 \pm 0.23$  |
| $\text{CAR}_R$ ( $\mu\text{g/g}$ DW) | ND               |
| CHLO ( $\mu\text{g/g}$ DW)           | $30.96 \pm 0.91$ |
| TEAC ( $\mu\text{mol TE/g}$ DW)      | $10.55 \pm 0.67$ |
| FRAP ( $\mu\text{mol TE/g}$ DW)      | $58.74 \pm 0.62$ |

TPC, total phenolic content; TF, total flavonoid;  $\text{CAR}_Y$ , yellow carotenoids;  $\text{CAR}_R$ , red carotenoids; CHLO, chlorophyll; TEAC, Trolox equivalents antioxidant capacity; FRAP, ferric reducing antioxidants power; GAE, gallic acid equivalents; QE, quercetin equivalents; TE, Trolox equivalents; ND, not detected. The data shown are the average of three independent replicates  $\pm$  SEM.

TABLE 3 Effects of dietary additives on performance of the laying hens over 12 weeks of feeding.

| Parameter | Dietary treatment   |                    |                    |                     |                     |       |         |
|-----------|---------------------|--------------------|--------------------|---------------------|---------------------|-------|---------|
|           | CON                 | ATB                | PRE                | AOX                 | PA                  | SEM   | P-value |
| BW (Kg)   |                     |                    |                    |                     |                     |       |         |
| T0        | 1.408               | 1.353              | 1.375              | 1.350               | 1.352               | 0.013 | 0.600   |
| T1        | 1.387               | 1.367              | 1.353              | 1.399               | 1.353               | 0.011 | 0.637   |
| T2        | 1.443               | 1.436              | 1.433              | 1.431               | 1.439               | 0.010 | 0.998   |
| ADFI (g)  |                     |                    |                    |                     |                     |       |         |
| T0        | 52.33               | 55.45              | 53.12              | 63.75               | 62.87               | 18.03 | 0.368   |
| T1        | 93.41               | 86.87              | 94.83              | 97.00               | 96.12               | 2.525 | 0.743   |
| T2        | 107.75              | 103.08             | 100.41             | 102.58              | 104.12              | 2.704 | 0.944   |
| FRC (g/g) |                     |                    |                    |                     |                     |       |         |
| T0        | 2.79                | 2.59               | 2.24               | 2.46                | 2.27                | 0.130 | 0.683   |
| T1        | 2.80                | 2.73               | 2.22               | 2.62                | 2.55                | 0.149 | 0.785   |
| T2        | 2.47                | 2.52               | 2.07               | 2.59                | 2.58                | 0.099 | 0.454   |
| EP (%)    |                     |                    |                    |                     |                     |       |         |
| T0        | 43.45               | 50.59              | 55.35              | 48.21               | 54.16               | 2.45  | 0.575   |
| T1        | 56.54               | 59.52              | 67.85              | 59.52               | 58.92               | 3.62  | 0.902   |
| T2        | 76.78               | 72.61              | 75.59              | 70.83               | 67.85               | 2.95  | 0.893   |
| EW (%)    |                     |                    |                    |                     |                     |       |         |
| T0        | 52.83               | 52.66              | 55.83              | 52.83               | 50.33               | 0.751 | 0.252   |
| T1        | 58.66 <sup>ab</sup> | 57.33 <sup>b</sup> | 64.50 <sup>a</sup> | 59.66 <sup>ab</sup> | 59.00 <sup>ab</sup> | 0.799 | 0.037   |
| T2        | 61.00 <sup>b</sup>  | 61.33 <sup>b</sup> | 68.66 <sup>a</sup> | 60.00 <sup>b</sup>  | 64.00 <sup>b</sup>  | 0.727 | 0.000   |
| EM (g)    |                     |                    |                    |                     |                     |       |         |
| T0        | 23.84               | 26.64              | 30.90              | 27.51               | 29.01               | 1.309 | 0.539   |
| T1        | 39.14               | 36.50              | 50.54              | 39.16               | 44.26               | 2.335 | 0.318   |
| T2        | 46.83               | 44.53              | 51.90              | 42.50               | 43.42               | 1.906 | 0.563   |

ADFI, average daily feed intake; EM, egg mass; EP, egg production; EW, egg weight. Data are presented as the means with their SEM (n=24). Different superscript letters indicate significant differences between the groups by Bonferroni's *post hoc* test ( $p < 0.05$ ). CON, basal diet without additive; ATB, basal diet + 0.02% colistin sulfate + 0.05% zinc bacitracin; PRE, basal diet + 0.5% agave fructans; AOX, basal diet + 0.5% turmeric powder; PA, basal diet + 0.5% agave fructans + 0.5% turmeric powder.

average daily feed intake (ADFI), feed conversion ratio (FCR), or egg production (EP) among treatments at any sampling time (T0, T1, or T2). In contrast, egg weight (EW) exhibited significant differences at T1 and T2 according to Bonferroni's *post hoc* test ( $p < 0.05$ ). At T1, hens supplemented with PRE showed higher EW values than those fed ATB diets, while at T2 the PRE group also maintained the highest EW relative to CON and other treatments. Egg mass (EM) increased numerically in the supplemented groups, although differences were not statistically significant ( $p > 0.05$ ). These results suggest that dietary inclusion of natural additives, particularly PRE supplementation, improved egg weight without affecting feed consumption or conversion efficiency.

Figure 1 provides a visual comparison of representative eggs obtained from hens under the different dietary treatments.

Table 4 presents the effects of the additives on external and internal egg quality traits at T0, T1, and T2. Most external parameters—egg length, egg width, egg shape index, and eggshell thickness—did not differ among treatments ( $p > 0.05$ ). However, EW differed at T1 ( $p = 0.037$ ) and T2 ( $p < 0.001$ ), with PRE hens showing higher values than the remaining treatments.

Regarding internal quality parameters, albumen height (AH) was significantly affected at T1 ( $p = 0.001$ ): PRE exhibited the highest AH, followed by CON and ATB, while PA showed the lowest. Yolk height (YH) also differed at T1 ( $p = 0.004$ ) and T2 ( $p < 0.001$ ). At both times, PRE and AOX showed higher YH than PA, indicating improved yolk structure. In addition, yolk width (YW) at T3 showed a statistically significant difference among treatments ( $p < 0.05$ ). Thin albumen (tA) at T2 showed a significant reduction in PRE and AOX compared with CON ( $p =$

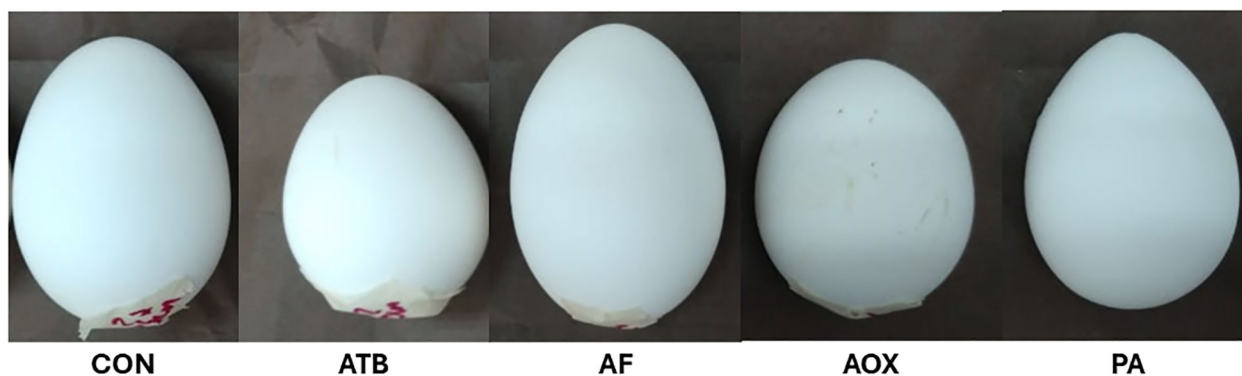


FIGURE 1

Representative eggs collected from hens after 12 weeks of dietary supplemented with a basal control diet (CON), antibiotics (ATB), agave fructans (PRE), turmeric powder (AOX), or the combination of both functional additives (PA).

TABLE 4 Effect of dietary additives on egg quality traits of the laying hens over 12 weeks of feeding.

| Traits              | Dietary treatment |                   |                    |                   |                   |       |                  |
|---------------------|-------------------|-------------------|--------------------|-------------------|-------------------|-------|------------------|
|                     | CON               | ATB               | PRE                | AOX               | PA                | SEM   | <i>p</i> - value |
| External parameters |                   |                   |                    |                   |                   |       |                  |
| ELT (cm)            |                   |                   |                    |                   |                   |       |                  |
| T0                  | 5.58              | 5.54              | 5.62               | 5.56              | 5.44              | 0.034 | 0.056            |
| T1                  | 5.81              | 5.65              | 5.88               | 5.68              | 5.66              | 0.028 | 0.082            |
| T2                  | 5.76              | 5.78              | 5.83               | 5.70              | 5.82              | 0.030 | 0.699            |
| EWD (cm)            |                   |                   |                    |                   |                   |       |                  |
| T0                  | 4.13              | 4.16              | 4.28               | 4.16              | 4.11              | 0.023 | 0.259            |
| T1                  | 4.25              | 4.29              | 4.42               | 4.33              | 4.28              | 0.118 | 0.129            |
| T2                  | 4.38              | 4.37              | 4.39               | 4.30              | 4.43              | 0.016 | 0.187            |
| ESI                 |                   |                   |                    |                   |                   |       |                  |
| T0                  | 74.2              | 75.11             | 76.1               | 75.0              | 75.7              | 0.414 | 0.667            |
| T1                  | 73.2              | 75.9              | 75.2               | 76.2              | 75.5              | 0.040 | 0.212            |
| T2                  | 76.1              | 75.6              | 75.3               | 75.4              | 76.0              | 0.297 | 0.938            |
| EST (mm)            |                   |                   |                    |                   |                   |       |                  |
| T0                  | 0.30              | 0.26              | 0.36               | 0.25              | 0.31              | 0.017 | 0.276            |
| T1                  | 0.37              | 0.35              | 0.35               | 0.42              | 0.37              | 0.065 | 0.391            |
| T2                  | 0.30              | 0.35              | 0.36               | 0.33              | 0.28              | 0.011 | 0.238            |
| Internal parameters |                   |                   |                    |                   |                   |       |                  |
| AH (mm)             |                   |                   |                    |                   |                   |       |                  |
| T0                  | 9.65              | 8.63              | 9.28               | 8.68              | 8.60              | 0.216 | 0.453            |
| T1                  | 9.30 <sup>a</sup> | 8.73 <sup>b</sup> | 10.93 <sup>a</sup> | 8.50 <sup>b</sup> | 6.68 <sup>c</sup> | 0.322 | 0.001            |
| T2                  | 9.75              | 9.61              | 9.11               | 7.65              | 7.55              | 0.280 | 0.055            |
| TA (mm)             |                   |                   |                    |                   |                   |       |                  |
| T0                  | 56.6              | 57.1              | 60.1               | 49.9              | 57.5              | 1.105 | 0.905            |

(Continued)

TABLE 4 Continued

| Traits              | Dietary treatment  |                    |                    |                    |                   |       |                 |
|---------------------|--------------------|--------------------|--------------------|--------------------|-------------------|-------|-----------------|
|                     | CON                | ATB                | PRE                | AOX                | PA                | SEM   | <i>p</i> -value |
| Internal parameters |                    |                    |                    |                    |                   |       |                 |
| T1                  | 56.9               | 60.6               | 61.1               | 62.4               | 60.1              | 0.717 | 0.153           |
| T2                  | 61.1               | 66.3               | 64.0               | 75.6               | 81.0              | 1.941 | 0.175           |
| TKA (cm)            |                    |                    |                    |                    |                   |       |                 |
| T0                  | 7.69               | 7.71               | 7.85               | 7.38               | 7.66              | 0.143 | 0.905           |
| T1                  | 7.78               | 7.61               | 7.91               | 8.36               | 8.31              | 0.105 | 0.085           |
| T2                  | 8.29               | 8.38               | 8.69               | 9.56               | 9.43              | 0.176 | 0.175           |
| EQI                 |                    |                    |                    |                    |                   |       |                 |
| T0                  | 123.9              | 121.72             | 123.7              | 126.5              | 124.7             | 0.798 | 0.456           |
| T1                  | 128.6              | 130.0              | 124.4              | 126.3              | 117.3             | 1.914 | 0.228           |
| T2                  | 116.3              | 114.1              | 117.9              | 117.1              | 113.5             | 0.773 | 0.314           |
| YH (mm)             |                    |                    |                    |                    |                   |       |                 |
| T0                  | 18.2               | 17.2               | 18.2               | 17.11              | 17.11             | 0.220 | 0.200           |
| T1                  | 18.4 <sup>ab</sup> | 18.1 <sup>ab</sup> | 19.0 <sup>a</sup>  | 19.0 <sup>a</sup>  | 17.8 <sup>b</sup> | 0.138 | 0.004           |
| T2                  | 19.5 <sup>a</sup>  | 19.4 <sup>a</sup>  | 17.8 <sup>ab</sup> | 16.2 <sup>b</sup>  | 15.2 <sup>b</sup> | 0.411 | 0.000           |
| YW                  |                    |                    |                    |                    |                   |       |                 |
| T0                  | 36.3               | 37.3               | 36.4               | 34.4               | 36.0              | 0.409 | 0.269           |
| T1                  | 38.4               | 37.7               | 40.4               | 40.1               | 45.5              | 1.063 | 0.154           |
| T2                  | 40.7 <sup>ab</sup> | 42.2 <sup>ab</sup> | 40.1 <sup>b</sup>  | 42.6 <sup>ab</sup> | 44.3 <sup>a</sup> | 0.478 | 0.047           |
| Color score         |                    |                    |                    |                    |                   |       |                 |
| T0                  | 4.66               | 4.00               | 6.00               | 5.83               | 5.83              | 0.237 | 0.166           |
| T1                  | 2.50               | 2.33               | 2.83               | 2.83               | 2.83              | 0.120 | 0.577           |
| T2                  | 2.16               | 2.33               | 2.00               | 2.00               | 1.83              | 0.135 | 0.834           |
| Yolk color          |                    |                    |                    |                    |                   |       |                 |
| L                   |                    |                    |                    |                    |                   |       |                 |
| T0                  | 57.4               | 58.5               | 58.1               | 58.4               | 57.6              | 0.302 | 0.799           |
| T1                  | 60.3               | 62.0               | 64.1               | 64.5               | 62.9              | 0.587 | 0.151           |
| T2                  | 64.3               | 65.1               | 67.2               | 66.0               | 65.4              | 0.471 | 0.404           |
| a*                  |                    |                    |                    |                    |                   |       |                 |
| T0                  | 6.60               | 6.65               | 6.58               | 5.54               | 6.05              | 0.291 | 0.730           |
| T1                  | 1.16               | 1.17               | 1.26               | 0.81               | 2.14              | 0.257 | 0.588           |
| T2                  | -0.94              | -0.71              | -0.67              | -1.83              | -1.49             | 0.151 | 0.043           |
| b*                  |                    |                    |                    |                    |                   |       |                 |
| T0                  | 81.6               | 77.1               | 78.2               | 74.6               | 77.3              | 1.374 | 0.634           |
| T1                  | 34.6               | 41.3               | 41.6               | 39.3               | 41.9              | 0.875 | 0.030           |
| T2                  | 32.1 <sup>a</sup>  | 28.6 <sup>ab</sup> | 31.6 <sup>ab</sup> | 28.8 <sup>ab</sup> | 25.5 <sup>b</sup> | 0.762 | 0.049           |

AH, albumen height; ELT, egg length; EQI, egg quality index; ESI, egg shape index; EST, eggshell thickness; ESW, eggshell weight; EWD, egg width; TA, thin albumen; TKA, thick albumen; YH, yolk height; YW, yolk width. Data are presented as the means with their SEM (n=6). Different superscript letters indicate significant differences between the groups by Bonferroni's post hoc test ( $p < 0.05$ ). CON, basal diet without additive; ATB, basal diet + 0.02% colistin sulfate + 0.05% zinc bacitracin; PRE, basal diet + 0.5 % agave fructans; AOX, basal diet + 0.5 % turmeric powder; PA, basal diet + 0.5 % agave fructans + 0.5 % turmeric powder.

0.049). No statistical differences were found in the egg quality index (EQI), color score (CS), or L\*, a\*, b\* color coordinates at any sampling point ( $p > 0.05$ ).

Table 5 shows the hematological and biochemical parameters of laying hens at the end of the feeding trial (T2). Erythrocyte counts were significantly higher in PRE compared with ATB and PA, while CON and AOX showed intermediate values ( $p < 0.05$ ). No significant differences were observed in hematocrit ( $p > 0.05$ ). Total leukocytes were significantly lower in AOX and PA compared with CON and PRE ( $p < 0.05$ ). The leukocyte profile was modified by dietary treatments: heterophils were significantly lower and lymphocytes significantly higher in PRE compared with CON and ATB ( $p < 0.05$ ). Consequently, the H:L ratio was significantly lower in PRE than in CON and ATB ( $p < 0.0001$ ). Monocyte percentages were higher in ATB than in AOX and PA ( $p < 0.05$ ). No significant differences were observed in eosinophils or basophils ( $p > 0.05$ ).

Regarding serum metabolites, glucose levels were lower in PA compared with CON and ATB ( $p < 0.05$ ). Calcium levels were also lower in PA compared with CON, ATB, and PRE ( $p < 0.05$ ). No significant differences were detected in phosphorus levels ( $p > 0.05$ ).

Supplementary Table S1 summarizes pH values, relative organ weights (ROW), and gastrointestinal tract lengths of hens at T2. In the crop, pH was significantly lower in ATB and PA than in CON ( $p < 0.05$ ), while ROW did not differ among treatments. In the gizzard, hens fed PRE had lower pH than those in ATB and AOX ( $p < 0.05$ ), although gizzard ROW did not differ. Duodenal ROW increased in AOX compared with ATB and PA ( $p < 0.05$ ), although pH remained unchanged. No significant differences were observed in pH or ROW in the proventriculus, jejunum, ileum, or large intestine. In the cecum, PRE hens exhibited higher pH values than ATB ( $p < 0.05$ ), while ATB showed reduced cecal ROW compared with CON, PRE, and AOX

( $p < 0.05$ ). The lengths of the small intestine, large intestine, and ceca were not affected by dietary treatments.

## 4 Discussion

In this study, we evaluated the combination of the antibiotics zinc bacitracin and colistin sulfate, commonly used in commercial poultry feed, particularly for laying hens and broilers. It is important to clarify that this antibiotic treatment was included solely as a conventional production benchmark, not as a recommended practice, in alignment with antimicrobial stewardship principles. The inappropriate and widespread use of antibiotics poses risks to consumer health, as residues may remain in animal-derived foods (Feng et al., 2016). These two antibiotics have been detected in milk and animal tissues in cattle (Wan et al., 2006), posing a risk of antimicrobial resistance in enterococcal infections in humans (Singer and Johnson, 2024). In this regard, recent research efforts to improve production parameters and egg quality in laying hens have focused on replacing antibiotics in animal feed. Diets enriched with functional ingredients improve production parameters and egg quality in laying hens (Vlaicu et al., 2021). These ingredients include antioxidants, organic acids, plant extracts, and oligosaccharides, among others (Goliomytis et al., 2019; Dilawar et al., 2021; Zhang et al., 2024, 2021). Moreover, there is evidence of the use of turmeric powder has been evaluated as a dietary additive for laying hens, both alone and in combination with prebiotics or other functional ingredients (Bozorgkhoo and Kuhi, 2025). In the present study, we proposed two natural dietary additives—agave fructans and turmeric powder—with well-documented functional properties demonstrated in *in vitro* assays and animal models as potential alternatives to antibiotics. The

TABLE 5 Effect of dietary additives on hematological parameters of the laying hens at the end of 12 weeks of feeding.

| Hematological parameters                   | CON                 | ATB                 | PRE                 | AOX                | PA                  | SEM   | P     |
|--------------------------------------------|---------------------|---------------------|---------------------|--------------------|---------------------|-------|-------|
| Erythrocytes ( $\times 10^6/\mu\text{L}$ ) | 3.40 <sup>a</sup>   | 2.45 <sup>ab</sup>  | 4.11 <sup>ab</sup>  | 3.12 <sup>ab</sup> | 2.95 <sup>b</sup>   | 0.17  | 0.020 |
| Hematocrit (%)                             | 18.63               | 19.00               | 20.13               | 17.88              | 18.13               | 0.40  | 0.438 |
| Leucocytes ( $\times 10^3/\mu\text{L}$ )   | 9.59 <sup>ab</sup>  | 8.38 <sup>ab</sup>  | 10.71 <sup>a</sup>  | 6.78 <sup>b</sup>  | 6.79 <sup>b</sup>   | 0.38  | 0.000 |
| Heterophils (%)                            | 20.22 <sup>a</sup>  | 21.77 <sup>a</sup>  | 13.11 <sup>b</sup>  | 16.67 <sup>b</sup> | 17.67 <sup>ab</sup> | 0.56  | 0.000 |
| Lymphocytes (%)                            | 71.67 <sup>b</sup>  | 71.11 <sup>b</sup>  | 79.44 <sup>a</sup>  | 76.89 <sup>a</sup> | 76.56 <sup>a</sup>  | 0.68  | 0.000 |
| H:L ratio                                  | 0.28 <sup>a</sup>   | 0.30 <sup>a</sup>   | 0.17 <sup>ab</sup>  | 0.22 <sup>b</sup>  | 0.23 <sup>b</sup>   | 0.00  | 0.000 |
| Monocytes (%)                              | 2.11 <sup>a</sup>   | 2.66 <sup>b</sup>   | 4.44 <sup>a</sup>   | 1.44 <sup>b</sup>  | 2.00 <sup>b</sup>   | 0.24  | 0.000 |
| Eosinophils (%)                            | 2.89                | 2.33                | 1.44                | 2.11               | 1.78                | 0.19  | 0.178 |
| Basophils (%)                              | 3.11                | 2.11                | 1.56                | 2.89               | 1.89                | 0.21  | 0.192 |
| <b>Biochemistry parameters</b>             |                     |                     |                     |                    |                     |       |       |
| Glucose (mg/dL)                            | 177.9 <sup>ab</sup> | 228.9 <sup>a</sup>  | 168.1 <sup>ab</sup> | 162.2 <sup>b</sup> | 143.4 <sup>b</sup>  | 8.08  | 0.003 |
| Calcium (mg/dL)                            | 19.24 <sup>a</sup>  | 14.85 <sup>ab</sup> | 17.3 <sup>ab</sup>  | 17.91 <sup>a</sup> | 6.17 <sup>b</sup>   | 0.977 | 0.000 |
| Phosphorus (mg/dL)                         | 11.29               | 10.17               | 11.57               | 10.19              | 8.83                | 0.722 | 0.636 |

Data are presented as the means with their SEM (n=10). Different superscript letters indicate significant differences between the groups by Bonferroni's *post hoc* test ( $p < 0.05$ ). CON, basal diet without additive; ATB, basal diet + 0.02% colistin sulfate + 0.05% zinc bacitracin; PRE, basal diet + 0.5% agave fructans; AOX, basal diet + 0.5% turmeric powder; PA, basal diet + 0.5% agave fructans + 0.5% turmeric powder.

selected doses were based on recent work by our research group (Franco-Robles and Hernández-Granados, 2024; Hernández-Granados et al., 2022; Guzmán-Rodríguez et al., 2024) to evaluate whether these additives, individually or in combination, could improve egg quality. The characterization of total bioactive compounds and *in vitro* antioxidant capacity in turmeric was consistent with values previously reported by (Yang et al., 2020; Pal et al., 2020). Regarding photosynthetic pigments, no CARR were detected; thus, total CAR consisted primarily of CARY pigments, mainly  $\beta$ -carotene,  $\beta$ -cryptoxanthin, and zeaxanthin (Hornero-Méndez and Mínguez-Mosquera, 2001). Similarly (Vasanthkumar et al., 2024), reported CAR values ranging from 2.78 to 17.32 mg/g and CHLO levels between 10 and 100  $\mu$ g/g in *Curcuma longa*.

Productive performance was not affected by the treatments, which aligns with previous studies where inulin-type fructans and turmeric supplementation do not compromise feed intake or hen productivity (Shang et al., 2010; Mosayyeb Zadeh et al., 2023). However, other reports have shown improvements in weight gain and feed consumption when agave fructans are supplemented for longer periods or at different physiological stages. For instance, Sánchez-Chiprés et al. (2021) observed enhanced weight gain from weeks 5 to 18 and increased feed consumption at weeks 15 and 20 in 36-week-old hens fed 0.1% of 0.2% agave fructans (Sánchez-Chiprés et al., 2021). Moreover (Shang et al., 2020), observed a 3.09% increase in laying rate and a 3.61% decrease in feed conversion ratio with inulin supplementation during feeding weeks 1 to 8. The contrast between these results may be due to differences in the degree of polymerization or botanical source of the fructans used (Mueller et al., 2016), as our formulation used a polydisperse mixture, in addition to the fact that the age of the hens was different (Gu et al., 2021). Moreover, this variability may depend on dosage, laying phase, and duration of supplementation.

In the present study, supplementation with 0.5% agave fructans improved egg weight and internal quality traits such as albumen and yolk height throughout the experimental period. These findings are consistent with previous reports indicating that inulin-type fructans can enhance nutrient utilization and egg mass. Shang et al., 2020 observed a 2.54% increase in egg weight with dietary inclusion of 15 g/kg inulin. Moreover, Obianwuna et al. (2022) demonstrated improvements in egg parameters after 84 days of supplementation with 0.3%–0.6% FOS, and Alsherify and Hassanabadi (2024) reported that 3.0 g/kg FOS improved the shape index and Haugh unit without affecting overall performance. Collectively, these results suggest that fructan-based prebiotics help preserve internal egg composition and structural integrity.

In contrast, the effects of dietary turmeric powder were less consistent, as the 0.5% inclusion did not produce quality improvements comparable to PRE. However, the relatively small number of eggs analyzed per treatment may have limited statistical power for detecting subtle effects in some internal quality traits. Future studies with larger sample sizes per replicate are therefore recommended. Similar dose-dependent responses have been reported previously, where turmeric levels below 2% reduced feed efficiency and negatively affected egg production and quality traits (Dalal and Kosti, 2018). Some studies report improvements at 0.5-

1% inclusion or turmeric powder while other studies found that the inclusion of 10 or 30 g/kg does not influence egg weight (Kosti et al., 2020). This variability indicates that higher doses or improved bioavailability of curcuminoids may be necessary to maximize the functional benefits of turmeric powder on egg quality.

To analyze the health status of the hens during supplementation with functional ingredients, we determined the hematological parameters. Bioactive dietary components are known to exert immunomodulatory effects that enhance resistance to pathogenic challenges in poultry (Obianwuna et al., 2022; Youssef et al., 2023). Our data showed that 0.5% agave fructans exhibited and increased total leucocyte count, accompanied by a reduction in heterophils and a higher proportion of lymphocytes resulting in a lower H:L ratio, an accepted indicator of animal stress (Franco-Robles and Hernández-Granados, 2024). These results suggest that agave fructans improve immune readiness and stress resilience in early-laying hens. Importantly, unlike mannanoligosaccharides (MOS), which have shown no changes in hematological profiles at dietary levels of 0.1–0.5 g/kg (Youssef et al., 2023), agave fructans elicited a clear immunomodulatory response. The effects of turmeric powder were more modest but still measurable, as reflected by a reduced H:L ratio compared with the control group. This finding is consistent with the known anti-inflammatory and antioxidant benefits of turmeric in laying hens (Kosti et al., 2020). Together, these results indicate that both agave fructans and turmeric enhance immune status, with the former displaying a more pronounced effect under the experimental conditions.

In this study, serum glucose levels were significantly higher in hens fed the antibiotic diet compared with all other treatments. Although further metabolic indicators were not assessed, this response may reflect metabolic stress associated with bacitracin exposure, as previously reported (Dowling, 2024). Regarding serum calcium, overall concentrations remained stable across diets except for the combination treatment (PA), which showed a significant decrease compared with the other groups. Prebiotics have been reported to improve calcium absorption and mineral homeostasis (Khan et al., 2020); therefore, the unexpected reduction observed in the PA group may indicate an interaction between fructans and turmeric that interferes with mineral uptake, warranting further investigation.

Finally, only limited gastrointestinal responses were observed following supplementation with 0.5% agave fructans and 0.5% turmeric powder. Diets containing PRE altered pH levels in specific intestinal segments, while AOX increased duodenal relative weight, indicating localized physiological responses in the upper digestive tract. These findings are consistent with previous reports suggesting that functional ingredients such as turmeric can influence digestive physiology and intestinal morphology in laying hens (Kosti et al., 2020). However, because changes were not consistent across all intestinal regions, the biological relevance of these effects remains uncertain. It is possible that the observed modifications are linked to microbial activity or fermentation processes in the gut, although this mechanism could not be confirmed in the present study. Thus, interpretations involving microbiota-driven effects should remain cautious until supported by microbial evidence in future investigations.

## 5 Conclusion

In summary, dietary supplementation with 0.5% agave fructans provided consistent physiological benefits in early-laying hens, improving egg weight and internal egg quality while favorably modulating hematological indicators of immune status, without compromising productive performance. Turmeric powder also contributed to a healthier immune profile, although its effects on egg quality were less evident at the evaluated dose. The combination of both additives did not outperform agave fructans alone.

Overall, these findings support agave fructans as a promising functional ingredient for improving egg quality and bird welfare, offering a viable alternative to conventional antibiotic growth promoters. Further research—including gut microbiota characterization and optimization of turmeric dosage and bioavailability—will be essential to fully elucidate the mechanisms involved and to refine combination strategies aligned with sustainable poultry production and One Health objectives.

## 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 approved by Comité de Ética para la Investigación de la Universidad de Guanajuato. The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

CBP: Conceptualization, Investigation, Methodology, Writing – review & editing. CGM: Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing. RCH: Conceptualization, Investigation, Methodology, Writing – review & editing. CO: Writing – review & editing. AGM: Formal analysis, Supervision, Writing – review & editing. EFR: Conceptualization, Investigation, Writing – original draft, Writing – review & 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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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fanim.2025.1688496/full#supplementary-material>

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