

Effects of acidifiers in the drinking water on performance, serum biochemistry, and digestive tract microbial counts in broilers

Heloisa Pagnussatt¹ , Gustavo Zaccaron¹ , Alicia Dal Santo¹ ,
Fernanda Danieli Antoniazzi Valentini¹ , Thainá Daiane Vogel¹ , Alan
Miranda Prestes¹ , Lilian Kolling Girardini¹ , Fernando de Castro
Tavernari² , Lenita Moura Stefani³ , Jean Kaique Valentim⁴ , Arele
Arlindo Calderano⁵ , Tiago Goulart Petrolli^{1*} 

¹ Universidade do Oeste de Santa Catarina, Programa de Pós-Graduação em Sanidade e Produção Animal, Xanxerê, SC, Brasil.

² EMBRAPA Suínos e Aves, Concórdia, SC Brasil.

³ Universidade do Estado de Santa Catarina, Chapecó, SC, Brasil.

⁴ Universidade Federal Rural do Rio de Janeiro, Instituto de Zootecnia, Departamento de Produção Animal, Seropédica RJ, Brasil.

⁵ Universidade Federal de Viçosa, Departamento de Zootecnia, Viçosa, MG, Brasil.

***Corresponding author:**

tiago.petroli@unoesc.edu.br

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ABSTRACT - This study evaluated the effects of supplementing drinking water with an acidifier blend on growth performance, serum biochemical profile, and gastrointestinal microbial counts of broilers. A mixture containing phosphoric, citric, and tartaric acids was supplied at 0, 60, or 150 mL/1000 L of drinking water during specific production phases. A total of 320 Cobb male broilers were allocated in a completely randomized design with eight replicates of 12 birds each. The acidifier progressively reduced drinking water pH values (5.54, 3.17, and 2.72, respectively). No effects ($P>0.05$) were observed on final body weight, feed intake, or feed conversion ratio. A quadratic trend ($P = 0.087$) was observed for total mesophilic bacterial counts in the crop, with the lowest point estimated at 103.87 mL/1000 L, whereas jejunal bacterial counts were not affected. Quadratic responses ($P<0.01$) were also observed for serum total protein, globulin, cholesterol, and uric acid concentrations, while albumin was not influenced ($P>0.05$). Supplementation of drinking water with a blend of citric, tartaric, and phosphoric acids at 150 mL/1000 L was safe, did not impair broiler performance, and maintained serum biochemical values within normal ranges. Quadratic responses among the evaluated concentrations (0, 60, and 150 mL/1000 L) suggest a potential inclusion range between 85 and 157 mL/1000 L associated with favorable outcomes. These findings indicate that the evaluated acid blend may contribute to gut health and represents a promising alternative to antibiotic growth promoters.

Keywords: citric acid, intestinal health, microbiota, phosphoric acid, tartaric acid

1. Introduction

Over the last few decades, growth-enhancing antibiotics have been used to ensure greater feed efficiency in broiler chickens. However, the inappropriate use of these antibiotics at subclinical doses has led to the prohibition of their use for this purpose, aiming to reduce potential risks to consumers,

thereby aligning with the concept of One Health. Residues of these compounds are frequently detected in products of animal origin, which can cause bacterial cross-resistance, impacting the efficiency of antimicrobial therapy for humans (Parent et al., 2020).

The poultry industry faces a series of sanitary challenges throughout chicken production, particularly concerning the intestinal health of birds. Gastrointestinal disorders, bacterial and parasitic infections, and the extensive use of antibiotics all negatively contribute to the microbial balance in the gastrointestinal tract of birds (Murshed et al., 2024). In this context, acidifiers, when added via drinking water, can help prevent gastrointestinal diseases (Adil et al., 2010) and boost immune system function, which results in improved growth performance and digestibility (Yadav and Jha, 2019).

Organic acidifiers provide energy, while inorganic acidifiers, such as phosphoric acid, offer nutrients such as phosphorus (Viola and Vieira, 2007), which play a crucial role in improving animal nutrition. Citric and tartaric acids, in particular, contribute to the integrity and stability of the intestinal microbiota by reducing pathogenic agents in the organism, which helps to decrease the occurrence of sanitary problems (Ángel-Isaza et al., 2019). These effects ultimately lead to improved bird performance, and both citric and tartaric acids are also safe for human health, making them potential substitutes for growth-promoting antibiotics (Ángel-Isaza et al., 2019; Saleem et al., 2020).

Phosphoric acid, in addition to its bactericidal effects on *Enterococcus faecalis*, has been shown to be more effective than citric acid (Arias-Moliz et al., 2008). It is one of the most commonly used acids in drinking water, not only for acidifying but also because it provides phosphorus for animal nutrition (Gao et al., 2021). Phosphoric acid is easily dissolved in water due to its ability to control pH (Lee et al., 2018) and remains stable at high temperatures with low volatility (Murahashi, 2009). Typically, it is found in an aqueous solution, which is colorless and can be corrosive depending on its dosage (Speight, 2017).

The use of acidifiers in the gastrointestinal tract lowers the pH, leading to physiological and morphological modifications that reduce the pathogenic microbial load, which in turn exert positive effects on the site's physiology and morphology, ultimately improving animal performance and immune function (Dittoe et al., 2018). Phosphoric acid specifically causes an immediate pH reduction in an aqueous medium, which helps it dissociate easily, thereby losing its main function of acidifying the bacterial cytoplasm. However, it still exerts beneficial effects by acidifying the intestinal lumen.

The hypothesis of this research is that supplementation of acidifiers through drinking water can reduce the microbial load in the digestive tract of broiler chickens without compromising the birds' performance and health. Therefore, the objective of this study was to evaluate the effect of the inclusion of a liquid blend of citric, tartaric, and phosphoric acids broiler performance, total mesophilic microbial counts in the crop and jejunum, and serum biochemical parameters in broiler chickens.

2. Material and methods

2.1. Animals, experimental design, and water treatment

This study was approved by the university's animal ethics committee under protocol number 03/2021.

This study was carried out at UNOESC - Poultry Sector facilities (Xanxerê city, SC, Brazil, Latitude: -26.836432730209985; Longitude: -52.40740389021019), using 320 male broiler chickens of the COBB lineage distributed on the first day of age in a completely randomized experimental design comprising three treatments (control, 60 mL and 150 mL of acidifier per 1000 L of drinking water) with eight repetitions each and 12 animals per experimental unit.

The animals were housed in 2 m² pens and raised according to the lineage manual, using shaving litter reused for five consecutive flocks, receiving feed (Table 1) in tubular feeders and nipple drinkers with water available *ad libitum* throughout the experimental period. The blends containing citric (5 - 10%), tartaric (5 - 10%), and phosphoric (60 - 80%) acids were added to nonchlorinated well water from 19 to 22 days, 26 to 28 days and from 33 until the end of the experiment (42 days of life). The broilers

were exposed to a light cycle of 16 hours of light and 8 hours of darkness, with a light intensity of 20 lux during light hours, in accordance with the animal welfare guidelines (Directive 2007/43/EC).

The birds were fed a balanced diet according to the nutritional guidelines recommended by Rostagno et al. (2017). The rations were provided *ad libitum*, with feeding occurring three times per day. The study period lasted from day 1 to 42 d of age, with feeding programs adjusted according to the growth stages of the birds (1-21 and 1-42 d).

Table 1 - Feed and nutritional composition of each reference diet of the experiment according to the rearing phase

Item	Starter (1-21 d)	Grower (21-32 d)	Finisher (32-42 d)
Ingredient			
Corn (g/kg)	476.66	508.25	651.16
Soybean meal (46%) (g/kg)	433.38	392.12	304.33
Soybean oil (g/kg)	45.90	61.85	48.96
Dicalcium phosphate (g/kg)	19.43	14.53	10.92
Limestone (g/kg)	8.20	8.35	7.14
Salt (g/kg)	4.55	4.25	4.07
DL-Methionine (99%) (g/kg)	3.55	2.60	2.54
L-Lysine HCl (g/kg)	2.19	3.16	2.22
Choline chloride (60%) (g/kg)	1.00	1.00	1.00
Vitamin supplement ¹ (g/kg)	2.00	2.00	2.00
Mineral supplement ² (g/kg)	2.00	2.00	2.00
L-Threonine	0.83	0.66	0.63
L-Valine	0.34	0.00	0.00
Calculated values		Amount	
Metabolizable energy (kcal/kg)	3050	3200	3250
Crude protein (g/kg)	243.00	226.20	195.40
Digestible lysine (g/kg)	13.63	12.35	10.67
Digestible met. + cys. (g/kg)	9.89	9.14	7.90
Digestible threonine (g/kg)	8.86	8.15	7.04
Digestible tryptophan (g/kg)	2.80	2.57	2.15
Digestible valine (g/kg)	10.29	9.51	8.22
Calcium (g/kg)	9.50	8.22	6.61
Available phosphorus (g/kg)	4.80	3.84	3.09
Sodium (g/kg)	2.25	2.11	2.01

¹ Vitamin supplement (per kg of product): vit. A - 10,000,000 IU; vit. D3 - 2,000,000 IU; vit. E - 30,000 IU; vit. B1 - 2.0 g; vit. B2 - 6.0 g; vit. B6 - 4.0 g; vit. B12 - 0.015 g; pantothenic acid - 12 g; biotin - 0.1 g; vit. K3 - 3.0 g; folic acid - 1.0 g; nicotinic acid - 50 g; selenium - 250 mg; and excipient qsp - 1000 g.

² Mineral supplement (per kg of product): iron - 100 g; cobalt - 2.0 g.

2.2. Drinking water pH

The drinking water pH was measured before and after acid dilution and is presented as an average for each treatment. This measurement was performed using a calibrated digital pH meter (Model HI 8424, HANNA Instruments®, Barueri, São Paulo, Brazil).

2.3. Performance

All animals were weighed weekly throughout the study to monitor growth and feed conversion ratio. Specifically, the broilers were weighed at 1, 21, and 42 d of age, along with the leftover feed, to determine the final weight (FW), feed intake (FI), and feed conversion ratio (FCR). Additionally, at 42 days of age, the productivity efficiency index (PEI) was calculated using the following formula: $PEI = (\text{Body weight} \times \text{Viability}) / (\text{Age at slaughter} \times \text{FCR})$.

2.4. Microbial analysis

At 42 d of age, one bird per experimental unit was humanely euthanized according to the guidelines of the National Council for the Control of Animal Experimentation (CONCEA, Brasil, 2013). The luminal content of the crop and jejunum was aseptically collected for microbial analysis and transported to the laboratory under refrigerated conditions. Samples were processed using the pour plate technique (Silva et al., 2010) using plate count agar (PCA, Oxoid). Briefly, aliquots of the luminal content were homogenized and serially diluted (up to 10^{-4}) in sterile saline solution (0.85%). From each dilution, 1 mL of inoculum was plated in duplicate. After incubation at 37 °C for 48 h, colonies were counted on plates containing 30 to 300 colonies, and the results were expressed as colony-forming units per gram of digesta (CFU g^{-1}).

2.5. Serum biochemical parameters

Blood samples (1 mL per animal at 42 d of age) were obtained from the brachial vein. Serum samples were obtained by centrifuging the blood at 3000 rpm and subsequently stored at -20 °C for analysis of total protein, albumin, globulin, cholesterol, and uric acid concentrations.

Biochemical analyses were carried out using specific commercial kits (Gold Analisa Diagnóstica Ltda., Belo Horizonte, MG, Brazil) for each parameter, which were measured using a Bioplus 2000 semi-automatic analyzer (Bioplus Produtos para Laboratórios Ltda., Barueri, SP, Brazil) at the Biochemistry Laboratory of the Department of Animal Science, State University of Santa Catarina (UDESC, Chapecó, SC, Brazil). Globulin levels were determined as the difference between total protein and albumin, and the results were expressed in g/dL of blood.

2.6. Statistical analysis

Experimental results were first assessed for normality using the Shapiro–Wilk test. Data meeting normality assumptions were subjected to analysis of variance (ANOVA) to identify significant differences among treatment groups. When appropriate, linear and quadratic regression analyses were applied to evaluate potential trends across the data. All statistical analyses were performed using R software (version 4.3.1), with significance established at a 0.05 probability level.

The pen was considered the experimental unit for performance-related variables (final weight, feed intake, feed conversion ratio, and production efficiency index). For serum biochemical parameters and microbiological analyses, one bird per pen was randomly selected, and thus the individual bird was considered the experimental unit for these variables.

$$Y_{ij} = \mu + \beta_i + \varepsilon_{ij}$$

in which Y_{ij} represents the dependent variables evaluated, such as final weight, feed intake, feed conversion, production efficiency index, total microbial count in the digestive tract (harvest and jejunum), and serum biochemical parameters (total protein, albumin, globulin, cholesterol, and uric acid); μ is the overall mean; β_i is the fixed effect of the treatment, represented by the different acidifier dosages (independent variable: 0, 60, or 150 mL/1000 L of water); ε_{ij} is the random experimental error associated with each observation.

3. Results

3.1. Drinking water pH

The average pH of the water used in the control group (without acid addition) was 5.54, while the pH values for the 60 mL/1000 L and 150 mL/1000 L acid treatments were 3.17 and 2.72, respectively.

3.2. Performance

The final body weight, feed intake (FI), feed conversion ratio (FCR), and productive efficiency index (PEI) were not affected ($P>0.05$) by the addition of liquid acids (Table 2).

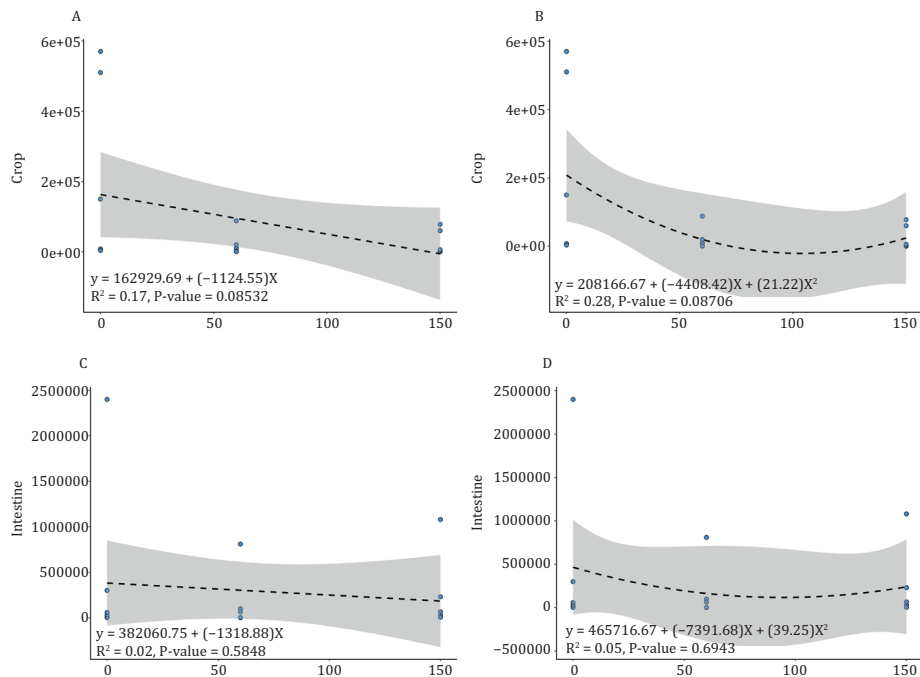
Table 2 - Performance of chicken broilers supplemented with acidifiers by drinking water

Variable	Control	60 mL/1000 L	150 mL/1000 L	Linear effect	Quadratic effect	CV (%)	SEM
1-21 days							
Weight (g)	969	1025	989	0.763	0.447	7.03	31.391
Feed intake (g)	1301	1342	1346	0.423	0.641	6.84	37.192
FCR	1.41	1.37	1.43	0.482	0.233	3.94	0.0233
1-42 days							
Weight (g)	3184	3268	3186	0.992	0.578	5.50	63.794
Feed intake (g)	4958	5042	4912	0.779	0.828	6.43	150.962
FCR	1.59	1.56	1.57	0.722	0.890	6.87	0.0413
PEI	459	474	456	0.863	0.821	13.57	22.168

FCR - feed conversion ratio; PEI - productive efficiency index; SEM - standard error of the mean.

3.3. Microbial analysis

In the control group (without acid addition), microbial counts of total mesophiles in both the crop and jejunum did not differ significantly ($P>0.05$; Figure 1). Comparatively, the birds that received acid treatments at 60 and 150 mL/1000 L exhibited similar microbial counts in the crop and jejunum, with a trend towards a quadratic reduction in microbial counts in the crop ($P = 0.087$). The quadratic curve reached its minimum point at a dosage of 103.87 mL/1000 L.



(A) Crop - linear regression; (B) Crop - quadratic regression; (C) Jejunum - linear regression; (D) Jejunum - quadratic regression. X-axis: Acidifier dose (mL/1000 L of water). Y-axis: Mesophilic bacterial count (log CFU/mL).

Figure 1 - Linear and quadratic regressions for total mesophilic bacterial counts in the crop and jejunum of 42-day-old broiler chickens receiving acidifiers via drinking water.

3.4. Serum biochemical parameters

The serum biochemical profile of the birds indicated a quadratic effect of acidifier doses on total protein ($P = 0.003$), globulins ($P = 0.001$), cholesterol ($P = 0.005$), and uric acid ($P < 0.001$) concentrations (Table 3). Additionally, a linear effect was observed for serum cholesterol ($P = 0.001$) and uric acid concentrations ($P < 0.001$) as the acidifier doses increased. No effects were found on serum albumin (ALB) concentration with acid supplementation at different doses ($P > 0.05$). The optimal acidifier concentrations, derived from the quadratic regression, were 86.84 mL/1000 L, 85.56 mL/1000 L, 157.20 mL/1000 L, and 125.16 mL/1000 L.

Table 3 - Serum biochemical parameters of chicken broilers supplemented with acidifiers by drinking water at 42 days of age

Variable	Control	60 mL/1000 L	150 mL/1000 L	CV	Linear effect	Quadratic effect	SEM
Total proteins (g/dL)	3.50	5.00	4.25	1.27	0.179	0.003 ¹	0.267
Albumins (g/dL)	1.87	1.71	1.69	0.37	0.272	0.445	0.102
Globulins (g/dL)	1.63	3.28	2.55	1.29	0.108	0.001 ²	0.266
Cholesterol (mg/dL)	64.00	72.71	78.14	12.98	0.001 ⁵	0.005 ³	2.724
Uric acid (mg/dL)	4.55	5.93	6.40	1.60	<0.001 ⁶	0.001 ⁴	0.318

SEM - standard error of the mean.

¹ $y = 3.49750 + (0.03821) X + (-0.00022) X^2$, $R^2 = 0.45728$, P -value = 0.003.

² $y = 1.63375 + (0.04155) X + (-0.00024) X^2$, $R^2 = 0.50810$, P -value = 0.001.

³ $y = 64.00000 + (0.17921) X + (-0.00057) X^2$, $R^2 = 0.42710$, P -value = 0.005.

⁴ $y = 4.55250 + (0.03004) X + (-0.00012) X^2$, $R^2 = 0.49996$, P -value = 0.001.

⁵ $y = 65.08814 + (0.09256) X$, $R^2 = 0.40243$.

⁶ $y = 4.77936 + (0.01198) X$, $R^2 = 0.43133$.

4. Discussion

The addition of liquid acids to drinking water demonstrated significant effects on reducing the microbial load in the upper digestive tract of birds without compromising productive performance (Ricke, 2019; Bourassa et al., 2018). This effect can be attributed to the mechanism of action of acids on the intestinal microbiota. As described by (Pickler et al., 2012), the antimicrobial action of acidifiers in the gastrointestinal tract depends on factors such as age, pH, oxygen level, acid concentration, site of action, and microbiome composition in each section of the tract. The effectiveness of acidifiers administered intermittently aligns with findings from similar studies, suggesting that continuous use throughout the production period may not be justified (Hamid et al., 2018).

According to Pickler et al. (2012), citric and tartaric acids have antimicrobial functions and can exist in either dissociated or non-dissociated forms (Russell, 1992). When combined with phosphoric acid, they help improve the intestinal microbiota when used in drinking water (Zhang et al., 2022). In the non-dissociated form, these acids carry protons (H^+), which, upon entering the bacterial cytoplasm, cause a decrease in pH, disrupting bacterial metabolism and resulting in bactericidal and bacteriostatic effects (Zhang et al., 2022; Dierick et al., 2002).

In this study, acids were administered in their free form (without encapsulation), which influenced their antimicrobial action primarily in the proximal sections of the digestive tract, with reduced impact on distal sections due to decreased acidification along the tract (Abdel-Fattah et al., 2008). Acidifiers are most effective in the crop, where there is no intrinsic pH control (Józefiak et al., 2010), leading to a greater reduction in bacterial populations. Supporting these findings, Van Bunnik et al. (2012) reported that adding acidifiers via drinking water increased acidity in the gizzard and stomach, thereby reducing bacterial presence throughout the gastrointestinal tract.

In the crop, phosphoric acid may play a key role in lowering pH immediately upon contact with the environment. The absence of endogenous secretions in this organ limits its natural acidification capacity,

making it more susceptible to the antimicrobial action of acids (Clavijo and Flórez, 2018). The use of acidified water during the rearing cycle reduces bacterial colonization in the upper gastrointestinal tract and subsequent sections (Hamed and Hassan, 2013; Mahmoud et al., 2012), contributing to a lower intestinal pH that inhibits harmful bacteria and promotes a balanced microbiota (Hamid et al., 2018).

Further studies have reported a reduction in bacterial load in the gizzard and gastrointestinal tract of broilers consuming acidified water (Van Bunnik et al., 2012). These findings suggest that the use of acidifiers in drinking water is safe for animal performance, as it does not compromise weight gain, feed intake, or feed conversion, although it does not enhance these parameters, as reported in other studies (Adhikari et al., 2020; Alçiçek et al., 2004). In contrast, positive performance effects in broilers were observed by Hu et al. (2020), suggesting that the reduced pH and increased proteolytic enzyme activity in the proventriculus improve nutrient digestion and exert bactericidal and bacteriostatic effects on pathogenic intestinal microorganisms. Acidifiers in drinking water may compensate for low endogenous acid production, often linked to metabolic disturbances, while controlling microbial load (Hamid et al., 2018).

At high doses, acids can limit water intake and even cause lesions in sensitive organs such as the tongue, esophagus, crop, gizzard, and proventriculus (Sugiharto et al., 2020). However, in this study, the dosages used were below commonly reported levels (200 mL/1000 L), and no lesions were observed at necropsy after 42 d. Mantzios et al. (2023) observed ulcers and lesions in the oral mucosa and esophagus with higher doses, along with reduced performance.

Reducing microbial load in broilers intended for slaughter is an important and ongoing challenge, as contamination increases carcass condemnation rates and poses a risk to public health (Peh et al., 2020). Pathogens such as *Campylobacter* spp. can colonize birds asymptotically but may contaminate carcasses and cause disease in humans.

In this experiment, total mesophilic bacterial counts in the crop showed greater reductions in groups receiving acidifiers compared with the control, demonstrating the antimicrobial efficacy of acids in the initial sections of the gastrointestinal tract. Citric acid, for example, reduced the pH to levels around 4.0, creating an environment that is unfavorable for pathogenic bacteria (Laury et al., 2009; Dan et al., 2007).

The water used came from an artesian well with a natural pH of 5.54, and the greatest microbial reduction was observed in group 2, with a final pH of 3.17 (60 mL/1000 L). Most pathogenic bacteria do not survive at pH values below 4.0, reinforcing the recommendation that acids achieve this range.

Although the primary goal is to eliminate harmful bacteria, some beneficial bacteria may also be affected, which should be minimized to preserve the beneficial microbiota (Ricke et al., 2020). The intestinal microbiota is essential for metabolism, productivity, and immunity in birds, as it competes with pathogens and exerts bactericidal and bacteriostatic effects (Clavijo and Flórez, 2018). The maintenance of local microbiota function is crucial to maximize nutrient absorption and support the biochemical processes of the digestive tract (Clavijo and Flórez, 2018).

Monitoring serum biochemical parameters is important for evaluating the efficacy of additives, as it provides insights into the general metabolic state of the birds. In this study, the serum biochemical profile indicated significant quadratic effects on total protein, globulins, cholesterol, and uric acid due to acid supplementation, with linear effects on serum cholesterol and uric acid. Globulins, key components of the immune system (immunoglobulins), increased with the use of acidifiers, suggesting an enhanced immune response (Campbell, 2004). This increase may also be related to inflammatory processes requiring elevated immunoglobulin production (Khan and Iqbal, 2016). Globulins are essential for immunity and dietary restrictions that reduce globulin levels may lead to immunosuppression (Ghazalah and Ali, 2008).

Total protein levels increased in relation to the albumin-to-globulin ratio (McKnight et al., 2020), with reference values of 3.23 and 3.27 g/dL. The control group showed higher values (3.50 g/dL), influenced by the acidifier dosage, with the highest value observed at 60 mL/1000 L (5.00 g/dL) and a slight decrease at 150 mL/1000 L (4.25 g/dL). Total protein levels increase as chickens age, reflecting changes

in phenotype and target tissue molecules (Tóthová et al., 2019; McKnight et al., 2020). While albumin levels remained stable, some studies suggest that acidifiers can reduce renal disorders in chickens (Harr, 2006). Changes in the albumin/globulin (A/G) ratio are age-dependent, being lower at 4 d and higher at 45 d, reflecting a balanced metabolism during growth (Tóthová et al., 2019).

Although cholesterol levels increased, they remained within the ranges described by Dierick et al. (2002) of 71.80 to 130 mg/dL, indicating that acidifier use did not affect liver or bile function, thereby ensuring bird welfare. Cholesterol plays a key role in the serum transport of fat-soluble vitamins (A, D, K, and E), bile acid synthesis, and structural integrity of cell membranes (Ludke and López, 1999).

Uric acid levels, indicative of renal function, were within safe limits, as renal dysfunction would increase concentrations (Wang et al., 2021). At high doses, acidifiers can stimulate renal production of free radicals, potentially damaging renal cells (Ghorbel et al., 2017).

The inclusion of acids in broiler drinking water showed promising results for reducing microbial load in the upper digestive tract without affecting productive performance. The antimicrobial effect of acids, particularly in the non-dissociated form, disrupts bacterial metabolism by depleting cellular energy and ultimately leading to bacterial death. The effectiveness in controlling pathogenic bacteria highlights the practical relevance of this method in the poultry industry, providing a positive perspective for gut health management in broilers.

5. Conclusions

Supplementation of drinking water with a citric, tartaric, and phosphoric acid blend (150 mL/1000 L) was safe, did not affect broiler performance, and maintained serum biochemical values within normal ranges. A trend toward reduced crop bacteria suggests potential gut health benefits, supporting its use as an alternative to antibiotic growth promoters.

Data availability

All data is available in the manuscript.

Author contributions

Conceptualization: Pagnussatt, H.; Zaccaron, G.; Dal Santo, A.; Valentini, F. D. A. and Vogel, T. D. **Data curation:** Prestes, A. M; Girardin, L. K.; Tavernari, F. C. and Stefani, L. M. **Funding acquisition:** Petrolli, T. G. **Investigation:** Petrolli, T. G. **Project administration:** Calderano, A. A. and Petrolli, T. G. **Writing – original draft:** Valentim, J. K.; Calderano, A. A. and Petrolli, T. G. **Writing – review & editing:** Valentim, J. K.; Calderano, A. A. and Petrolli, T. G.

Conflict of interest

The authors declare no conflicts of interest.

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