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# Intestinal morphometric changes associated with the use of non-antibiotic feed additives in broiler chicks challenged with *Salmonella* Enteritidis

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## Abstract

Non-antibiotic feed additives stand as a potential alternative for antimicrobial growth promoters, but their effects in the gastrointestinal tract of broiler chicks suffering early infection are poorly understood. This study aimed to investigate the effects of two non-antibiotic feed additives (a postbiotic and a sanguinarine-based phytobiotic) on the gut morphology and body weight gain of broiler chicks challenged with *Salmonella enterica* serovar Enteritidis (SE). Birds (n = 144) were distributed according to a 2 × 3 factorial in a completely randomized design with the following treatments: non-challenged chicks fed control diet (SHAM-DCO), postbiotic (SHAM-PFC), or sanguinarine-based compound (SHAM-SAN) and SE-challenged chicks fed control diet (SE-DCO), postbiotic (SE-PFC), and sanguinarine-based compound (SE-SAN). Birds from each treatment were euthanized at 3-, 7-, and 14-days post inoculation and samples were collected for SE counting and intestinal morphometry. Weight gain was determined at 14 days post-inoculation. Lower ($p \leq 0.05$) *Salmonella* counts were observed in birds fed diets containing PFC at 3- and 7-days post inoculation. SE-challenged chicks showed greater crypt depth ($p \leq 0.05$) and lamina propria thickness ($p \leq 0.05$) and smaller villus:crypt ratio ($p \leq 0.05$) at the different sampling periods. Overall, birds fed PFC or SAN showed decreased lamina propria thickness ($p \leq 0.05$), greater villus height ($p \leq 0.05$), villus:crypt ratio ($p \leq 0.05$), and larger villus area ($p \leq 0.05$) compared with those fed the control diet (DCO). SAN supplementation improved body weight ($p \leq 0.05$) and weight gain ($p \leq 0.05$) until 14 days post-hatch compared with the control diet. Both feed additives (PFC and SAN) improved birds' response to post-hatch *Salmonella* Enteritidis infection, evidenced by beneficial changes in gut morphology. These effects highlight the potential of these feed additives to improve gut health of broiler chicks during the initial rearing phase.

**Keywords:** Antibiotic alternatives, Broilers, Feed additives, Postbiotic, Sanguinarine Salmonellosis

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**Availability of data and material**
Upon reasonable request, the datasets of this study can be available from the corresponding author.

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**Ethics approval and consent to participate**
All management, slaughter and sampling procedures were approved by the Ethical Committee of Animal Use in Research of the Federal University of Paraíba (Comissão de Ética no Uso de Animais da Universidade Federal da Paraíba) under the protocol number CEUA 140-17. The protocols are in compliance with the regulations established by the National Council for the Control of Animal Experimentation (CONCEA, Brazil) by means of the Law No. 11.794/2008 (the Arouca Law), and the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments).

## INTRODUCTION

Considering the global threat to public health posed by the emergence and dissemination of antimicrobial resistant bacteria [1], the animal industry has been moving towards the reduction in the use of antimicrobial drugs, especially performance enhancers, also known as antimicrobial growth promoters (AGPs), which are added to animal feed at low concentrations to promote growth [2]. The use of AGPs in food animals was banned in the European Union since 2006 and has been significantly reduced in other regions, particularly for AGPs belonging to antimicrobial classes that are related to highest priority critically important antimicrobials (HPCIAs) in human medicine [3]. Although there has been intense debate from both science and policy perspectives about the extent to which the use of antibiotics in food animals can contribute to the development of antimicrobial resistance in human pathogens [4–6], there is accumulated scientific evidence [7–9] suggesting that the use of AGPs is contributing to the emergence and dissemination of antimicrobial-resistant bacteria, and that their use will likely be further restricted or banned in the future [10].

Non-antibiotic feed additives such as postbiotics and sanguinarine-based phytobiotic emerged as alternative solutions to AGPs for performance enhancing purposes [11,12] due to their anti-inflammatory activity and capacity to modulate the immune system [13,14]. However, most results originated from experiments under ideal or favorable production conditions. On the other hand, there is a lack of studies addressing the effects and mechanisms of action of non-antimicrobial growth feed additives under challenging conditions, such as infectious agents. *Salmonella enterica* subsp. *enterica* (*S. enterica*) is a leading foodborne agent worldwide [15] and serovar Enteritidis remains as a major problem for public health, and particularly for the poultry industry [16] because of the frequent human salmonellosis outbreaks attributed to the consumption of poultry meat and eggs [15,17]. *Salmonella enterica* serovar Enteritidis (SE)-contaminated eggs were the cause of the largest known salmonellosis outbreak in Europe, resulting in 1,209 reported cases across 16 different countries between 2015 and 2018 [18]. Broiler chickens are more susceptible to SE infection during the post-hatching period because the intestinal microbiota is not fully established, and the immune system is still under development [19].

We hypothesized that non-antibiotic feed additives can improve intestinal morphology and mitigate *Salmonella* Enteritidis colonization in broiler chicks and improve performance. Therefore, this study investigated the effects of a postbiotic and a sanguinarine-based phytobiotic on cecal SE counts, ileum morphometry and weight gain in SE-challenged chicks.

## MATERIALS AND METHODS

All management, slaughter and sampling procedures were previously approved by the Ethical Committee of Animal Use in Research of the Federal University of Paraíba (Comissão de Ética no Uso de Animais da Universidade Federal da Paraíba) under the protocol number CEUA 140-17. The protocols follow the regulations established by the National Council for the Control of Animal Experimentation (CONCEA, Brazil) by means of the Law No. 11.794/2008 (the Arouca Law), and the ARRIVE guidelines (Animal Research: Reporting of *in vivo* Experiments).

### Experimental design

A total of 200 fertile eggs weighing 69 ± 2.9 g from 31-week-old-age Cobb500 were incubated at 37.7℃ and 60% relative humidity in a commercial incubator with hourly automatic turning cycle (IP130, Premium Ecológica). Eggs were candled at 10 days of incubation to discard infertile eggs

and dead embryos. After hatching, chicks were weighed individually, and cloacal swabs were taken for *S. enterica* screening.

Following standardization of body weight (mean = 48.4 g), a hundred forty-four males and females were distributed according to a 2 × 3 factorial in a completely randomized design with six treatments and two pens per treatment (n = 12 per pen). Birds were individually identified with leg bands and kept in solid-floored pens (0.8 m × 0.8 m) with a minimum area of 0.05 $m^2$ per bird, and 0.4 m height from one to 14 days of age. Pens were covered with nylon mosquito screens to avoid vector-borne *S. enterica* cross-contamination. Feed and water were provided *ad libitum* throughout the experiment, and the length of feed trough was at least 7 cm per bird. An initial phase ground diet was formulated with 22.4% crude protein, 1.32% digestible lysine, 0.95% methionine + cysteine, 1.94% glycine+serine and 0.86% digestible threonine [20]. The feed additives were added to the feed according to the manufacturers' recommendations (1.25 g/kg postbiotic; 50 mg/kg of commercial product containing ≥ 1.5% sanguinarine). The postbiotic (Original XPC, Diamond V) is composed of fermentation metabolites of *Saccharomyces cerevisiae* yeast grown on media of processed grain by-products, roughage products, cane molasses, malt and corn syrup [21]. It also contains yeast cell wall fragments, such as mannooligosaccharides and β-glucans. The sanguinarine-based phytobiotic (Sangrovit, Phytobiotics Futterzusatzstoffe GmbH) is an herbal preparation derived from the plant *Macleaya cordata* containing the biologically active substances sanguinarine (≥ 1.5%), as the predominant alkaloid compound, and cheleritrine (≥ 0.75%) [22]. The six treatments included non-challenged chicks fed control diet, i.e., without additives (SHAM-DCO), SE-challenged chicks fed control diet (SE-DCO), non-challenged chicks fed postbiotic fermented compound (SHAM-PFC), SE-challenged chicks fed postbiotic ferment compound (SE-PFC), non-challenged chicks fed sanguinarine-based compound (SHAM-SAN), and SE-challenged chicks fed sanguinarine-based compound (SE-SAN).

Individual weight gain was calculated by the difference between final and initial weights and results were expressed as mean and standard deviation values for each treatment.

## Bacterial strain, challenge, and euthanasia

Birds were challenged with a nalidixic-acid resistant *Salmonella* Enteritidis strain ($SE^{Nal+}$). An aliquot (100 µL) of a fresh $SE^{Nal+}$ culture was transferred to 40 mL nutrient broth (Neogen) and incubated at 37℃ for 24 hours in an orbital shaker. The inoculum was serially diluted (1:10) and from each dilution three 20 µL-drops were placed onto brilliant green agar (BGA) plates containing nalidixic acid (100 µg/mL). After incubation at 37℃ for 24 hours, colonies were counted, and values were expressed in colony-forming units per mL (CFU/mL).

Hatchlings were inoculated in the crop at one day post-hatching with 0.5 mL of nutrient broth (sham-inoculated groups) or nutrient broth containing 8.3 × $10^7$ $SE^{Nal+}$ (SE-inoculated groups) using 14-gauge bent crop-feeding needle. Six chicks per treatment were randomly weighed and euthanized by cervical dislocation at 3-, 7-, and 14-days post-inoculation.

## Microbiological procedures

Cloacal swabs were taken from all birds at day 0 (before inoculation) for *S. enterica* screening. The swabs were placed into nutrient broth (Neogen) supplemented with nalidixic acid (100 µg/mL) and incubated at 37℃ for 24 h. A 20-µL aliquot was spread onto BGA (Neogen) plates also supplemented with nalidixic acid (100 µg/mL).

Cecal contents were collected from the euthanized birds at 3-, 7-, and 14-days post-inoculation for $Salmonella^{Nal+}$ counting according to the drop plate method as previously described [23]. Shortly, the contents were weighed and then serially diluted (1:10) in buffered peptone water (Neogen).


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SE enumeration was performed similarly to the inoculum counting and values were expressed in colony-forming units per gram of cecal content (CFU/g).

### Morphometric analyses

Ileal gut samples of approximately 3 cm were collected from four animals in each sampling day. The samples were washed with 0.9% NaCl and fixed in 10% formaldehyde for 24 hours. Subsequently, the samples were dehydrated using a series of alcohol solutions (70%, 80%, 90% and 100%), cleared with xylol and embedded in paraffin. Semi-serial sectioning (5 μm) was performed in microtome (Hyrax M25, Zeiss) and 5 to 7 sections were placed on each slide. Two slides were prepared for each sampled animal. The slides were stained with hematoxylin and eosin and analyzed under light microscopy. Villus height (VH), crypt depth (CD), villus:crypt ratio (V:C), villus area (VA), and thickness of lamina propria (LP) were measured using Image J [24]. VH was measured from its apex to the basal region, which coincides with the surface of the crypt. Crypts were measured from the region of transition between the crypt and the villus and crypt basis. The thickness of LP was measured from the crypt region to the muscular layer of the mucosa. Villus width (VW) was measured at the medial portion of the villus. For each morphometric variable, ten measurements were performed in samples from four animals per treatment, resulting in 40 replicates. V:C was calculated using VH and CD. VA was determined using VW and VH, according to the equation described by Sakamoto et al. [25]: $(2\pi) \times (VW / 2) \times (VH)$.

### Statistical analyses

Morphometric measurements and performance data were evaluated in a completely randomized experimental design according to a 2 × 3 factorial, considering as main factors inoculation (sham- or SE-inoculated) and diet (DCO, PFC or SAN). Performance parameters (initial weight, final weight, and weight gain) were assessed using 10 birds per treatment, with each bird being considered a replicate. Analyses were performed using a commercial statistical software (Sisvar version 5.6, UFLA). Differences between means were assessed by Tukey test at 5% significance level of probability.

## RESULTS

No *Salmonella* spp. was detected in hatchlings before inoculation (day 0) or in the cecal contents of sham-inoculated birds at 3, 7, and 14 days. *Salmonella* $^{Nal+}$ was recovered from all (6/6) SE-inoculated birds at day 3 from groups DCO, PFC and SAN; at day 7, *Salmonella* $^{Nal+}$ was detected in all six birds in group DCO but only in five (5/6) birds in each PFC and SAN groups. Lower *Salmonella* $^{Nal+}$ counts ($p \leq 0.05$) were observed in birds fed diets containing PFC at 3- and 7-days post-inoculation, as shown in Table 1. *Salmonella* $^{Nal+}$ was detected in only 1/6, 1/6 and 2/6 birds from groups DCO, PFC and SAN at day 14, respectively. No mortality was recorded in sham- or SE-inoculated birds throughout the experimental period.

At 3 days post inoculation, there was no significant interaction ($p \leq 0.05$) between the main factors for VA, therefore, considering inoculation and diet separately. VA was not affected by inoculation, but it was larger ($p \leq 0.05$) in PFC- and smaller ($p \leq 0.05$) in DCO-fed animals. At the same age, interaction ($p \leq 0.05$) was observed for all other morphology variables (Table 2). Considering both SE- and sham-inoculated groups, intestinal mucosa development was greater in animals fed either PFC or SAN, with greater VH, CD and V:C ($p \leq 0.05$) (Table 2). In addition, SE-inoculated birds, regardless of dietary supplementation, reduced VH and V:C ratio ($p \leq 0.05$) compared with sham-inoculated birds. Interestingly, PFC or SAN supplemented diets reduced LP ($p$

**Table 1.** Qualitative testing (positive animals/total of animals) and mean cecal bacterial counts (CFU/g) in broilers challenged with *Salmonella* Enteritidis$^{Nal+}$ and fed control diet (DCO), diet supplemented with sanguinarine (SAN) and diet containing a postbiotic fermented compound (PFC) at 3, 7 and 14 days post-hatching

| Treatment | 3 days | | 7 days | | 14 days[1)] |
|---|---|---|---|---|---|
| | Positive/total | Cecal counts (CFU/g) | Positive/total | Cecal counts (CFU/g) | Positive/total |
| DCO | 6/6 | 9.01 ± 0.41[a] | 6/6 | 6.23 ± 0.94[a] | (1/6) |
| SAN | 6/6 | 8.28 ± 0.86[a] | 5/6 | 6.38 ± 0.61[a] | (2/6) |
| PFC | 6/6 | 7.99 ± 0.73[b] | 6/6 | 5.11 ± 0.35[b] | (1/6) |

[1)]Only qualitative Salmonella testing was performed on day 14 post-hatching.

[a,b]Means followed by the same letters in the columns are similar by Tukey test a 5% probability.

**Table 2.** Villus height (VH), crypt depth (CD), villus:crypt ratio (V:C), and thickness of lamina propria (LP) in broiler chicks fed basal diet (DCO), or diet supplemented with a postbiotic fermented compound (PFC) or sanguinarine-based compound (SAN) at 3 days post-inoculation (3 dpi) with *Salmonella* Enteritidis (SE) or nutrient broth (Sham)

| | DCO | PFC | SAN | *p*-value |
|---|---|---|---|---|
| VH (μm) | | | | |
| Sham | 290.47 ± 7.9[aB] | 332.40 ± 10.0[aA] | 320.66 ±12.0[aB] | < 0.001 |
| SE | 281.46 ± 22.3[aB] | 308.94 ± 16.5[bA] | 307.98 ± 14.4[aA] | 0.02 |
| *p*-value | 0.11 | 0.045 | 0.08 | |
| CD (μm) | | | | |
| Sham | 74.81 ± 1.8[aB] | 92.03 ± 2.9[bA] | 78.58 ± 4.4[bAB] | < 0.001 |
| SE | 74.96 ± 4.9[aB] | 107.31 ± 16.5[aA] | 102.89 ± 10.9[aA] | < 0.001 |
| *p*-value | 0.47 | 0.03 | < 0.001 | |
| V:C (μm: μm) | | | | |
| Sham | 3.88 ± 0.1[aA] | 3.92 ± 0.2[aA] | 3.61 ± 0.2[aA] | 0.23 |
| SE | 3.75 ± 0.1[aA] | 3.18 ± 0.4[bB] | 2.92 ± 0.3[bB] | 0.04 |
| *p*-value | 0.14 | 0.047 | 0.02 | |
| LP (μm) | | | | |
| Sham | 19.11 ± 1.0[bA] | 16.54 ± 1.6[bB] | 15.68 ± 2.2[bB] | 0.04 |
| SE | 27.32 ± 0.7[aA] | 20.87 ± 2.2[aB] | 20.58 ± 1.5[aB] | 0.02 |
| *p*-value | < 0.001 | 0.01 | 0.01 | |

Mean values followed by the same small letters in the columns or capital letters in the row are similar by Tukey test a 5% probability.

≤ 0.05) both in sham- and SE-inoculated animals (Table 3). An increase in LP ($p \leq 0.001$ for DCO and $p \leq 0.01$ for PFC and SAN) was observed in all groups challenged with *Salmonella* regardless of diet.

No interaction ($p \geq 0.05$) was observed at 7 days (Table 3) post-inoculation for any of the morphology parameters and thus, means are presented considering the two main factors separately (inoculation and diet). At seven days post-inoculation, SE-challenged chicks showed increased CD and LP ($p \leq 0.05$) and decreased V:C ratio ($p \leq 0.05$) (Table 3). Regarding the diets, PFC-birds showed decreased LP ($p \leq 0.01$) compared with DCO-fed birds. Both PFC-and SAN-fed birds had greater VH, V:C ratio, and larger VA ($p \leq 0.05$) compared with DCO-fed birds (Table 3). Greater VH, and V:C ($p \leq 0.05$) were observed in PFC-birds compared with SAN-fed birds.

There was no interaction between diet and inoculation ($p \leq 0.05$) at 14-days post-inoculation for CD, V:C, and LP (Table 4). Therefore, means are presented considering the two main factors separately (inoculation and diet). V:C was smaller ($p \leq 0.05$) in birds inoculated with *Salmonella*. Birds fed PFC or SAN diets had greater CD ($p \leq 0.01$) but smaller V:C ($p \leq 0.001$) compared with


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**Table 3.** Villus height (VH), crypt depth (CD), villus:crypt ratio (V:C), and thickness of lamina propria (LP), and villus area (VA) in broiler chicks fed basal diet (DCO), or diet supplemented with a postbiotic fermented compound (PFC) or sanguinarine-based compound (SAN) at 7 days post-inoculation (7 dpi) with *Salmonella* Enteritidis (SE) or nutrient broth (Sham)

| | VH (μm) | CD (μm) | V:C (μm:μm) | LP (μm) | VA (μm) |
|---|---|---|---|---|---|
| Inoculation | | | | | |
| Sham | 387.29 ± 27.4[a] | 84.25 ± 8.6[b] | 4.59 ± 0.6[a] | 20.87 ± 2.4[b] | 0.15 ± 0.02[a] |
| SE | 383.06 ± 25.7[a] | 98.53 ± 12.1[a] | 3.88 ± 0.3[b] | 26.69 ± 1.7[a] | 0.14 ± 0.01[a] |
| Diet | | | | | |
| DCO | 336.84 ± 22.4[c] | 88.05 ± 3.7[a] | 3.82 ± 0.4[c] | 25.45 ± 2.0[a] | 0.12 ± 0.02[b] |
| PFC | 428.02 ± 28.5[a] | 95.06 ± 10.2[a] | 4.50 ± 0.6[a] | 21.34 ± 1.1[b] | 0.17 ± 0.01[a] |
| SAN | 390.67 ± 19.3[b] | 92.40 ± 5.8[a] | 4.22 ± 0.4[b] | 23.37 ± 2.5[ab] | 0.15 ± 0.02[a] |
| *p*-value | | | | | |
| Inoculation | 0.15 | 0.04 | 0.02 | 0.03 | 0.35 |
| Diet | 0.02 | 0.34 | 0.04 | 0.01 | 0.02 |
| Inoculation × Diet | 0.09 | 0.12 | 0.07 | 0.09 | 0.06 |

[a–c]Within each factor, means followed by the same letters in the columns are similar by Tukey test a 5% probability.

**Table 4.** Crypt depth (CD), villus:crypt ratio (V:C) and thickness of lamina propria (LP) in broiler chicks fed basal diet (DCO), or diet supplemented with a postbiotic fermented compound (PFC) or sanguinarine-based compound (SAN) at 14 days post-inoculation (14 dpi) with *Salmonella* Enteritidis (SE) or nutrient broth (Sham)

| | CD (μm) | V:C (μm:μm) | LP (μm) |
|---|---|---|---|
| Inoculation | | | |
| Sham | 125.95 ± 18.4[a] | 4.21 ± 0.6[a] | 26.51 ± 2.1[a] |
| SE | 136.04 ± 16.8[a] | 3.40 ± 0.5[b] | 25.26 ± 2.8[a] |
| Diet | | | |
| DCO | 98.78 ± 20.5[b] | 4.55 ± 0.9[a] | 26.67 ± 1.2[a] |
| PFC | 145.15 ± 11.5[a] | 3.64 ± 0.2[b] | 22.36 ± 3.4[b] |
| SAN | 149.06 ± 18.5[a] | 3.42 ± 0.5[b] | 26.11 ± 2.9[a] |
| *p*-value | | | |
| Inoculation | 0.09 | 0.046 | 0.34 |
| Diet | 0.01 | 0.00 | 0.03 |
| Inoculation × Diet | 0.11 | 0.08 | 0.13 |

[a,b]Within each factor, means followed by the same letters in the columns are similar by Tukey test a 5% probability.

DCO-fed birds. PFC-supplemented diet reduced LP ($p \leq 0.05$) compared with other treatments (Table 4).

At 14 days post-inoculation, there was interaction ($p \leq 0.05$) between the main factors for VH and VA (Table 5). VH was reduced in SE-inoculated birds regardless of diet ($p \leq 0.01$ for PFC and $p \leq 0.001$ for DCO and SAN). Independent of inoculation treatment, PFC-fed birds had larger VA compared to animals fed DCO or SAN (Table 4).

No interaction ($p \geq 0.05$) was observed for final weight and weight gain for the period from 1 to 14 days of age (Table 6). There was no difference ($p \geq 0.05$) between SE-inoculated and sham-inoculated birds for those performance variables. Considering the factor diet, the final weight and weight gain of animals fed SAN were higher ($p \leq 0.05$) than DCO. The weight gain of PFC-fed animals was not different from DCO and SAN (Table 6).

**Table 5.** Villus height (VH) and villus area (VA) in broiler chicks inoculated with *Salmonella* Enteritidis (SE) or nutrient broth (Sham) at 14 days post-inoculation (14 dpi) under different dietary treatments: basal diet (DCO), diet supplemented with a postbiotic fermented compound (PFC) or sanguinarine-based compound (SAN)

| | DCO | PFC | SAN | *p*-value |
|---|---|---|---|---|
| VH (μm) | | | | |
| Sham | 461.75 ± 12.3$^{aB}$ | 567.62 ± 25.0$^{aA}$ | 554.18 ± 11.7$^{aA}$ | < 0.001 |
| SE | 431.80 ± 11.0$^{bB}$ | 495.88 ± 6.2$^{bA}$ | 458.0 ± 19.4$^{bB}$ | < 0.001 |
| *p*-value | < 0.001 | 0.01 | < 0.001 | |
| VA (μm) | | | | |
| Sham | 0.17 ± 0.02$^{aB}$ | 0.30 ± 0.03$^{aA}$ | 0.17 ± 0.01$^{aB}$ | < 0.001 |
| SE | 0.15 ± 0.01$^{aB}$ | 0.20 ± 0.01$^{bA}$ | 0.16 ± 0.01$^{aB}$ | 0.04 |
| *p*-value | 0.06 | 0.02 | 0.11 | |

Mean values followed by the same small letters in the columns or capital letters in the row are similar by Tukey test a 5% probability.

**Table 6.** Initial weight (g/bird), final weight (g/bird), and weight gain (g/bird) of broiler chicks (1 to 14 days) fed basal diet (DCO), or diet supplemented with a postbiotic fermented compound (PFC) or sanguinarine-based compound (SAN) and inoculated with *Salmonella* Enteritidis (SE) or nutrient broth (Sham)

| | Initial weight (g/bird) | Final weight (g/bird) | Weight gain (g/bird) |
|---|---|---|---|
| Inoculation | | | |
| Sham | 48.50 ± 1.5$^{a}$ | 423.42 ± 43.6$^{a}$ | 374.91 ± 43.4$^{a}$ |
| SE | 48.39 ± 2.5$^{a}$ | 407.27 ± 44.1$^{a}$ | 358.89 ± 49.1$^{a}$ |
| Diet | | | |
| DCO | 48.37 ± 2.1$^{a}$ | 381.65 ± 51.5$^{b}$ | 333.27 ± 51.8$^{b}$ |
| PFC | 48.42 ± 1.8$^{a}$ | 413.32 ± 51.9$^{ab}$ | 364.80 ± 50.9$^{ab}$ |
| SAN | 48.52 ± 2.2$^{a}$ | 451.07 ± 37.3$^{a}$ | 402.64 ± 36.0$^{a}$ |
| *p*-value | | | |
| Inoculation | 0.97 | 0.22 | 0.21 |
| Diet | 0.98 | 0.04 | 0.03 |
| Inoculation × Diet | 0.90 | 0.56 | 0.58 |

$^{a,b}$Within each factor, means followed by the same letters in the columns are similar by Tukey test a 5% probability.

## DISCUSSION

According to our results, PFC-fed broilers had lower SE counts in cecal while SAN supplementation improved body weight and weight gain until 14 days post-hatch compared with the control diet. Moreover, either PFC or SAN significantly improved bird response to post-hatch SE infection, evidenced by improved gut morphology.

The lower SE counts observed in birds fed diets containing PFC corroborates previous reports [26,27]. Lower SE counts after PFC treatment is possibly associated with the reduced colonization due to the presence of mannooligosaccharides (and their breakdown products, such as D-mannose) and β-glucans that bind to pathogenic bacteria inhibiting their adhesion to enterocytes [28]. In-feed mannooligosaccharides [29] and D-mannose added to drinking water [30,31] significantly reduced *Salmonella* colonization in broilers. Besides directly binding to pathogenic bacteria, these compounds can also module the immune system contributing to the maintenance of a healthy intestinal environment [32,33].

We observed no statistically significant reduction in SE counts in birds fed SAN, even though previous studies have reported reduced cecal *Salmonella enterica* counts in broiler chickens fed diets


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supplemented with this compound [34–36]. However, it should be noted that our study is restricted to the post-hatching phase, differing from those studies addressing the whole production cycle.

Greater LP thickness at all sampling periods in SE-challenged birds could be associated with inflammation, characterized by increased leukocyte infiltration, villus atrophy and crypt hyperplasia as a response to the continuous immune stimulation [37]. The mucosal damage caused by pathogenic bacteria colonization exposes toll-like receptors that are present in the LP to their ligands in the gut lumen, such as lipopolysaccharides, peptidoglycan, and flagellin [38]. Interestingly, DCO-fed birds had greater LP thickness, suggesting that both PFC and SAN ameliorated the inflammatory signs associated with SE infection. Changes in morphology such as thickened LP can compromise absorption of nutrients and the production of mucins, increasing the susceptibility to infections. [39]. LP thickness can also be associated with proinflammatory microbial populations due to dysbiosis [40] and the effects of PFC and SAN on the gut microbiome of broiler chickens should be further investigated. According to the available literature, the beneficial effects of SAN on the gut morphology of broiler chickens were associated with increased Firmicutes abundance and reduced the pro-inflammatory cytokines tumor necrosis factor α (TNF-α) and interleukin (IL)-4 in jejunum mucosal [14].

Sanguinarine has been shown to cause anti-inflammatory effects in both *in vitro* and *in vivo* studies, possibly related to a decrease in the secretion of TNF-α [13,41]. As a quaternary benzo[c] phenanthridine alkaloid, sanguinarine shows an irreversibly inhibitory influence on intestinal aromatic amino acid decarboxylase, thus reducing the production of biogenic amines [42]. Furthermore, the impact of using phytochemical compounds on meat safety must be investigated, as it has been also associated with positive effects on broiler carcass and meat quality [42–44].

Similar effects have been also observed in cells exposed to yeast fermentation products due to internalization of metabolites with high antioxidant capacity and inactivation of free radicals [45]. These fermentation metabolites can also improve the immune response by stimulating the expression of the cytokines, such as CD69 and CD25, on natural killer (NK) and natural killer T (NKT) cells, increasing the cytotoxic response and the proliferation of B cell populations [32].

Villus height (VH) is an important morphometric parameter due to the absorptive function of the brush border in the villus apex [46]. Increased VH observed at 3, 7 and 14 days post-inoculation in birds from both PFC and SAN groups might indicate a beneficial effect in terms of intestinal epithelium renewal, which is determined by the balance between cell loss at villus apex and enterocyte production by crypts [47]. Thus, smaller CD associated with greater VH is usually indicative of less injury and consequently, less cell turnover in the villi. Therefore, our results suggest that both additives (PFC and SAN) improved intestinal health, corroborating previous studies [14,36,45,48,49]. The beneficial effects of PFC- or SAN-supplemented diets on the intestinal morphology of chicks could be observed as early as 3 dpi, which is expected considering the high rate of intestinal cell turnover at this stage, as indicated by Yamauchi [50]. Moreover, the post hatching period correlates with a higher susceptibility to *Salmonella* colonization [39], possibly explaining the marked differences in gut morphology observed between SHAM and SE-inoculated birds.

Although increased VA and VH were observed in PFC- compared with SAN-supplemented birds, only the latter had significantly greater weight gain. Pickler et al. [34] have also reported improved weight gain in SAN-fed birds, even though no changes in VH were observed. The enhanced performance of animals fed sanguinarine could be attributed to its anti-inflammatory activity and capacity to modulate gut microbiota [51,52]. Increased Firmicutes/Bacteroidetes ratio was observed in SAN-fed chickens [14]. Such modulation, also reported for metabolites of yeast fermentation, is driven by increased concentrations of short-chain fatty acids (SCFA)

such as acetate, propionate, butyrate, and valerate, promoting upregulation of beneficial acid-lactic bacteria [53].Therefore, the improvement in the performance of birds fed SAN seems to be associated with reduced mucosal challenge by gut bacteria, and therefore lower energy expenditure, since the maintenance of active immunity in animals is energetically costly and may compromise performance [54]. Considering that Firmicutes are more effective as an energy source than Bacteroidetes, increased Firmicutes/Bacteroidetes ratio improves carbohydrate absorption and, consequently, weight gain [55]. Moreover, increased growth in animals fed sanguinarine has been attributed to modulating effects on the Trp-serotonin pathway leading to increased feed intake [42].

In conclusion, the non-antibiotic feed additives evaluated in this study showed beneficial effects on the intestinal health of sham- and *Salmonella* Enteritidis-inoculated hatchlings during the initial phase. Both PFC and SAN ameliorated the inflammatory response triggered by post-hatch *Salmonella* Enteritidis infection. In non-infected birds, however, PFC significantly improved gut morphology. Moreover, this additive significantly reduced *Salmonella* gut colonization during post-hatching. On the other hand, the use of SAN favored weight gain during the initial phase compared with other treatments. Our findings corroborate the empirical evidence suggesting that commercial non-antimicrobial feed additives might represent feasible alternatives to AGPs in the poultry industry. This is particularly important in a scenario in which the use of antimicrobials as growth promoters has been significantly reduced.

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