

Stevia as a natural additive on gut health and cecal microbiota in broilers

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ABSTRACT

Stevia mash (SM), leaves of *Stevia rebaudiana* Bertoni plant, is an additive used in poultry that enhances growth and health. Objective: to determine the effect of 1 % SM on productive parameters, gut health, and the cecal microbiome in broilers between the first 15 and 21 days old. One hundred sixty male, 1-day-old broilers (48.5 ± 2.5 g) were divided into Control (C) without SM and Treated (T) with 1 % SM on diet, during 15/21 days. Each subgroup had eight broilers/five repetitions/treatment. At day 15 or 21, all broilers were dissected, Fabricius Bursa and Gut removed and processed for histomorphometry, followed by Villi Height/Crypt Deep (VH/CD) ratio. Conversion Index (CI) was determined. The V3-V4 region of 16S rRNA gene was amplified from DNA obtained from pooled cecal contents and sequenced on Illumina Miseq PE 2 × 250 platform. Sequence processing and taxonomic assignments were performed using the SHAMAN pipeline. Both T groups have better VH/CD Ratios than C groups ($p \leq 0.05$). In guts, increased plasmatic and goblet cells number and thicker mucus layer were found in T15 and T21. All groups received SM showed early immunological maturity in Fabricius Bursa. IC was similar between all treatments. *Faecalibacterium*, *Ruminococcus torques* group, and *Bacteroides* were the major genera modulated by SM addition. At 15 and 21 days old, SM exerts an impact on diversity and evenness of the cecal microbiome. Conclusion: SM (1 %) produced early immunologic maturity on Fabricius Bursa, increased intestinal functionality, and modified the microbiota, increasing beneficial microbial genera and microbial diversity.

1. Introduction

In intensive broiler production, the objective is to obtain high-performance productivity at a low cost. To accomplish production goals and satisfy the increased demand for poultry meat worldwide, intense genetic selection, health, management, and nutrition programs have emerged (Peralta et al., 2016). As a major development priority for nutrition, researchers are looking for natural additives to replace antibiotic growth promoters (AGPs). The AGPs were banned since their use was associated with antimicrobial resistance and the presence of residues in feed and environment together with the preferences of avian consumers who look for natural products (Mehdi et al., 2018; Peralta et al., 2019, 2020; Korver, 2022; Ducatelle et al., 2023).

On the avian nutritional intervention, research is focused on targeting to evaluate the enhancement of gut health. The term gut health refers to the maintenance of the harmonic interaction of the microbiota-

brain-gut axis. This axis is intricately connected within the gut, involving the gut-associated lymphoid tissue (GALT), microbiota, and enteric nervous system. These components interact and influence each other through the release of various biological substances. Subsequently, incorporating natural products into the diets that regulate this axis during the initial 15-21 days, a critical period of its development, holds significance (Peralta et al., 2016; Kogut, 2019; Taha-Abdelaziz et al., 2018; Tellez-Isaías and Latorre, 2022). These natural products are probiotics, prebiotics, enzymes, phytobiotics, etc (Mehdi et al., 2018; Kogut, 2019; Peralta et al., 2019; Korver, 2022). Inside phytobiotics, Stevia (*Stevia rebaudiana* Bertoni) (S) is used as a sweetener in humans, but it contains bioactive compounds with other properties less known like antioxidant, antimicrobial, antitumor, antifungal, and positive modulator on the immunologic system (Quesada-Figueroa, 2011; Christaki et al., 2013; Peralta et al., 2018a; Xu et al., 2023).

In animal productions, S is added to diets in different forms: stems,

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leaves, or extracts from leaves. They contain different bioactive compounds, mainly steviosides and rebaudiosides (Christaki et al., 2013). In poultry, S addition to broiler diets is associated with enhanced productive performance, gut health, and humoral immunology. For example, S Extract (SE) added to diets or water (0.5-1 %) for 15 days enhanced productive efficiency and increased gut health and humoral immunology in broilers (Quesada-Figueroa, 2011; Peralta et al., 2018b; 2020; Raheem et al., 2022). S mash (SM) added to broiler diet (0.5-2 %) for 15 days resulted in early immunological maturation of Fabricius Bursa and increased gut health (Vaquero et al., 2022). Improvements in production efficiency and gut health, measured at 49 days were reported in broilers when fed SM (0.5-2 %) for the first 21 days (Quesada-Figueroa, 2011). In another study, the addition of SM or pure steviosides (0.13 %) for 4 weeks increased weight gain in broilers during the first 15 days, but did not change productive efficiency at the end of the trial (at 4 weeks) (Atteh et al., 2008, 2011). It seems that the S effect over productive parameters is noticed when this phytobiotic is added to the diets of broilers during the first 15-21 days. Although the mechanism related to the S-positive effects is still being studied, reports on the effect of SM on the microbiota are lacking. Changes in the diversity of the cecal microbiome, mainly inside the first 15-21 days old, could have significant effects on nutrient absorption and utilization, disease resistance, and overall health and performance productivity of broilers (Kogut, 2019; Taha-Abdelaziz et al., 2018; Korver, 2022; Tellez-Isaías and Latorre, 2022; Kasti et al., 2022). Changes in the diversity of the cecal microbiome, mainly inside the first 15-21 days old, could have significant effects on nutrient absorption and utilization, disease resistance, and overall health and performance productivity of broilers (Pourabedin & Zhao, 2015; Kogut, 2019; Taha-Abdelaziz et al., 2018; Korver, 2022; Tellez-Isaías and Latorre, 2022; Kasti et al., 2022). The determination of modifications in the microbiota induced by S supplementation allows the understanding of the positive or negative effect exerted on beneficial bacterial genera that interact with both gut and GALT to enhance the growth performance, gut functionality, and gut health into the early age (first 15-21 days old) in broilers. (Peralta et al., 2019, 2020, Ducatelle et al., 2023). The objective of this study was to determine the effect of Stevia mash (SM) (Stevia rebaudiana Bertoni) (1 %) on productive parameters, gut health, and the cecal microbiome in broilers between the first 15 and 21 days of age. In addition, we compared the effect of SM on the variables mentioned on days 15 and 21 to determine the shortest time when the effects become evident.

This study provides compelling evidence for the use of SM as a natural additive in poultry production during the critical developmental period between 15 and 21 days of age. It is well-established that SM enhances both growth productivity and overall health in poultry, potentially offering a viable alternative to the use of antibiotics as growth promoters.

2. Materials and methods

2.1. Poultry and management

One hundred sixty male, 1-day-old Ross 308 broiler chicks weighing 48.5 ± 2.5 g were used. On the 18th incubation day, all eggs were vaccinated against Newcastle, Marek, and Infectious Bursal Diseases (live) and on day one, the chicks were vaccinated against Infectious Bronchitis and Coccidiosis Disease (live). Chicks were housed in pens, in Avian Research Unit at the UNRC.

2.2. Stevia preparation

The S harvest was carried out, at the Plant Production Unit of the Development Laboratory of the UNRC when plants reached the pre-flowering phenological stage, by cutting at 0.10 m of the soil surface preserving buds for regrowth. Plants were dried on tables in a dry and airy place, in the absence of direct sunlight. The dried leaves were milled

with an analytical mill, using meshes between 40 and 200 μ m and then SM was added to the broiler's diet at corresponding levels (Vaquero et al., 2022). Proximal analysis of SM is showed in Table 1.

2.3. Experimental design and dietary treatments

All the experimental procedures applied in this study were reviewed and approved by the by the National University of Rio Cuarto (UNRC) Ethics Committee (Protocol Project: Foncyt 3120/2019) and conducted in accordance with the Guidelines for Experimental Animals (Olfert, et al., 1993).

One hundred sixty males, one day broiler chicks Ross 308 were used from birth to fifteen-twenty-one days. Chicks were housed in the Avian Research Unit, at the UNRC, Rio Cuarto, Córdoba, Argentina (33°06'35"S 64°18'07"W). This Unit is equipped with four battery cages with five cages (60 × 40 × 50 cm) (replicates) each battery, to accommodate the broilers for one to 15/21 days (with an air conditioning system to control the temperature and manual feeders and nipple drinkers inside each cage). All chicks were weighed on day one and distributed randomly into cages receiving the different treatments. Four distinct treatment groups were established for this study: Control (C) without SM for days 1 to 15 (C15); Control, without SM for days 1 to 21 (C21); Treated (T) with 1 % SM added to diet, for days 1 to 15 (T15); Treated with 1 % SM added to diet, for days 1 to 21 (T21). Each treatment group of 40 chicks was subdivided into 5 replicates composed of 8 chicks each (4 × 5 × 8- Treatment x replicates x chicks). The allocation of the treatments was done in a completely randomized manner to ensure unbiased distribution. Feed and water were offered *ad libitum*. Broilers received a pre-started diet from day 1 to 7 and a starter diet from day 8 to 15 or 21. Diets were formulated according to Aviagen (2012) and Rostagno (2017). The basal composition of the pre-start and starter diets used in trials is shown in Table 2. They were elaborated in the Feed Balanced Unity into Avian Research Unity at the UNRC.

2.4. Performance measurement and sampling

During the experimental period from beginning until end (1-15 or 1-21 days old) total weight of the broiler for each pen was obtained. In the same way, all food added to the feeder in each pen was registered to measure consumption. Broiler mortality was recorded and mortality percentage was determined at the end of the study.

The productive variables measured were: Average Daily Gain (ADG) (g/bird/day), Average Daily Consumption (ADC) (g/bird/day) and Conversion Index (CI). ADG was obtained as final-initial total weight/pen from each treatment divided 8 (broiler number inside each pen) divided 15 or 21 days. ADC was obtained as consumption registered in each pen divided 8 (broiler number inside each pen) divided 15 or 21 days. CI was obtained as consumption divided total weight broilers in each pen (Peralta et al., 2018).

2.5. Immunological and gut health variables

At the end of the experiment (15 or 21 days), immediately following euthanasia, all broilers were dissected. Fabricius bursa and gut samples

Tables 1
Composition of Stevia Mash.

Nutrient level	%
Protein	11.5
Humidity	7.0
Ashes	7.1
Crude fiber	12.5
Fat	3.8
Carbohydrates	37.3
Steviosides and Rebaudiosides	9.6
Others	11.2

Table 2
Composition and Nutrient Levels of Basal Diet (g Ingredient/Kg Diet) (as Feed).

Ingredients	Pre-starter (1-7 days old)	Starter (8-21 days old)
Corn	506	566
Soybean meal	357.3	210
Full fat soy (heat-treated)	60	150
Meat flour (45)	55	55
Vitamin and mineral premix ¹	5	5
NaCl	4	3
D-L methionine	4	3
Lysine	4	3
Split shell	4.7	5
Total	1,000	1,000
Nutrient levels		
Crude protein	240	214
Calcium	9.5	9.5
Crude fat	4	5
Crude fiber	2	2.5
Lysine	14	12.5
Methionine	6	5.5
Tryptophan	2.9	2.3
Metabolic energy, kcal/kg	2,950	3,150

¹ Vitamin Mineral premix Provided the following per kilogram of diet: vitamin A (retinol), 4.05 mg; vitamin D3 (cholecalciferol), 0.0875 mg; vitamin E (alpha-tocopherol), 45 mg; vitamin K3 (menadione), 3 mg; vitamin B1 (thiamine), 3.25 mg; vitamin B2 (riboflavin), 7.5 mg; vitamin B6 (pyridoxine), 5 mg; vitamin B12 (cyanocobalamin), 0.0325 mg; biotin, 0.15 mg; Ca-pantotenate, 15 mg; niacin, 45 mg; folic acid, 1.5 mg; choline chloride, 600 mg; Mn (MnSO₄), 100 mg; Zn (ZnO), 75 mg; Fe (FeSO₄), 67.5 mg; Cu (CuSO₄), 17.5 mg; I (KI), 1 mg; and Se (Na₂SeO₃), 0.275 mg (Aviagen, 2012 and Rostagno, 2017).

were collected for histological and histomorphometric analysis, by conventional methodology, stained with hematoxylin-eosin, and examined by optical microscopy. For this study, Axiophot microscope (Carl Zeiss, Germany) equipped a digital camera (AXio Cam Erc 5x Rev.2, Carl Zeiss, Germany) was used (Peralta et al., 2018).

The histomorphometric variables measured on gut were: Villus Height (VH) (μm), Crypt Depth (CD) (μm), and VH/CD. The VH/CD was obtained by using Blue Program (provided by Carl Zeiss with the AXio Cam) and observing a minimum of 20 fields per histological section/broiler. Then Image J program was used for the measurement, taking a minimum of 20 fields per histological section/broiler (Peralta et al., 2018).

2.6. DNA extraction and 16S rRNA gene sequencing

Cecal content was collected and pooled from all broilers of each treatment (40 broilers per treatment). DNA from the pooled cecal samples was extracted using a QIAamp® PowerFecal® Pro DNA kit (QIAGEN, Germany), quantified using a NanoDrop One Spectrophotometer (Thermo Scientific), and sent to the sequencing service provided by the Institute of Agrobiotechnology and Molecular Biology (IABIMO-CONICET-INTA, Buenos Aires, Argentina). The V3-V4 region of the bacterial 16S rRNA gene was amplified, and 250 bp paired-end sequencing was carried out on an Illumina Miseq PE 2 × 250 platform.

2.7. OTU building process and taxonomic assignment

Sequence processing and taxonomic assignments were performed using the SHAMAN pipeline (Volant et al., 2020). 511,389 amplicons were recovered from the 16S rDNA region. After the removal of singletons and chimera amplicons, the SHAMAN pipeline clustered the 5,794 remaining amplicons in OTUs using a cutoff value of 97 % similarity. Thus, 235 OTUs were obtained and annotated against the SILVA database using SHAMAN. Abundance and weighted non-null normalized count tables were generated using SHAMAN.

2.8. Statistical analysis

All the data derived in this study were checked for normal distribution prior to performing statistical analysis. Productive and gut health data were analyzed by ANOVA (completely randomized design), using Infostat software® (2016) (Di Rienzo et al., 2016).

ANOVA analysis was applied with two factors: the first factor encompassed the treatments, including two levels (control and SM), and the second factor involved the ages, with two levels (15 and 21 days). Mathematical model for ANOVA (completely randomized design):

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

Where: Y_{ij}: observation of treatment i in pen j

τ_i: effect of treatment i

ε_{ij}: random error term associated with observation Y_{ij}.

The modified Shapiro-Wilk test was used to assess normal distribution. When ANOVA showed differences between means, the Least Significant Difference (LSD) test was applied. Significant values were declared if $P < 0.05$, and $0.05 < P < 0.10$ was considered a tendency.

3. Results

3.1. Performance

In this study we observed that SM (1 %) on diet had no changes in productive variables; hence broilers 15 and 21 days old had the same IC, consumption, and weight gain as controls. (Table 3). No mortality was observed in this assay.

3.2. Gut health and immunologic variables

Gut histomorphometric analysis revealed a noteworthy significantly increase in the VH/CD ratio in all broilers that received SM ($p \leq 0.05$) both at 15 and 21 days. This increase was observed because in both groups receiving SM, the CD significantly decreased and VH significantly increased in comparison to the control group ($p \leq 0.05$) (Table 4).

During the histological analysis, we observed that broilers receiving SM (T15 and T21) exhibited an increased number of goblet cells and their secretions, including mucin, which is a component of the gut mucus layer. Furthermore, abundant plasmatic cells (P) responsible for producing IgA were observed in the SM groups. In T21, plasmatic cells were observed near the apical border of the villi (Fig. 1)

In this study, the Fabricius bursa displayed early development in all broilers fed SM (T15 and T21) (Fig. 2). Additionally, it was observed that broilers at 21 days old exhibited more advanced development compared to those at 15 days, both in the control and SM groups.

Table 3

Productive variables in broilers fed Stevia Mash during 15 and 21 days.

	Treatments 15 days			Treatments 21 days		
	Control	SM	P value	Control	SM	P value
ADG ¹	22.90 ± 0.3	23.49 ± 0.2	0.204	44.39 ± 0.4	44.22 ± 0.3	0.346
ADC ²	29.50 ± 0.4	29.69 ± 0.2	0.285	44.50 ± 0.6	44.83 ± 0.4	0.211
CI ³	1.08 ± 0.0	1.06 ± 0.0	0.912	1.23 ± 0.0	1.22 ± 0.0	0.890

¹ ADG: Average Dairy Gain.

² ADC: Average Dairy Consumption.

³ CI: Conversion Index.

Treatments: Control: without SM, and SM: with 1 % Stevia mash. Treatment duration: 15 and 21 days.

Table 4
Gut health variables on broilers fed Stevia Mash during 15 and 21 days.

Treatments	Control		P value	Stevia mash		P value
	15 days	21 days		15 days	21 days	
VH (μm)	701489 \pm 13890 a	812234 \pm 10790 b	0.424	812234 \pm 10790 a	839682 \pm 11998 b	0.455
CD (μm)	100496 \pm 3431 a	90264 \pm 1321 b	0.483	100524 \pm 1245 a	96275 \pm 989 b	0.478
VH/CD	7.01 a	8.08 b	0.416	7.83 a	8.72 b	0.489

VH: Villus Height; CD: Crypt Depth; VH/CD: Villus Height/Crypt Depth.
Treatments: C: without SM, and T: with 1 % Stevia mash, with the treatment duration: 15 and 21 days.

Different letters indicate significant differences, ($P < 0.05$).

3.3. Cecal microbiome analysis

The effect of SM addition on the cecal microbiome of broilers at two different ages, 15 and 21 days old, was examined. Using 16S rDNA MiSeq sequencing, we further characterized the microbiota composition of cecal samples pooled from chickens control and those that received 1 % SM at 15 and 21 days old. The SHAMAN pipeline (Volant et al., 2020) was used to analyse amplicons from the microbial community. The samples consisted of 511,389 amplicons, with a median length of 403 bp. After dereplication, which involved the identification and removal of duplicate sequences, the number of amplicons was reduced to 344,415. Singleton removal was performed, resulting in 7,975 remaining amplicons. Finally, chimera removal was performed, which involved identifying and removing sequences that were the result of PCR artifacts. After this step, the final number of amplicons was 5,794. Using these sequences, 235 operational taxonomic units (OTUs) were identified.

The bar plot (Fig. 3A) and the box plot (Fig. 3B) show the proportions

of relative abundance to normalized counts from the most abundant genera established in the cecal content. The relative abundance of the genus *Faecalibacterium* was greater with SM addition at 15 days of age but then decreased at 21 days of age. *Faecalibacterium* is a beneficial bacterial genus in the gut and an important member of the cecal microbiome which has been linked to various health benefits. For example, *Faecalibacterium* produces short-chain fatty acids that have anti-inflammatory and immunomodulatory effects. The *Ruminococcus torques* group is another major genus modulated by SM addition at 21 days of age. *Ruminococcus* species are important for fiber degradation in the gut and also are known to produce short-chain fatty acids. In contrast, the abundance of the bacterial genus *Bacteroides* changed dramatically with SM addition, with opposing results: it increased at 15 days of age but dramatically decreased at 21 days of age.

Negativibacillus, a bacterial genus isolated from the guts of animals including poultry, was found to have a greater relative abundance at 21 days of age, independently of the diet. The genera *Lachnospirillum*, *Eisenbergiella*, *Colidextribacter*, *Oscillibacter*, and *Sellimonas* were also important components of the gut microbiome and were found to have significantly increased relative abundance with SM addition at 21 days of age. The relative abundance of *Parabacteroides* increased significantly in the control group at 21 days of age but was not detected as one of the majority genera at 15 days of age. The relative abundance of *Anaerotruncus* was similar in both diets, and remained stable over time. The stable relative abundance of *Anaerotruncus* in both groups may indicate that it is a resident bacterium unaffected by SM addition.

Finally, SM addition significantly affected the relative abundance of *Escherichia-Shigella* in the cecal microbiome of chickens. At 15 days of age, the relative abundance of *Escherichia-Shigella* was significantly lower in the treatments group than that in the control group.

The diversity of the cecal microbiome was evaluated using the Shannon and Simpson indexes (Fig. 4). The Shannon index measures the

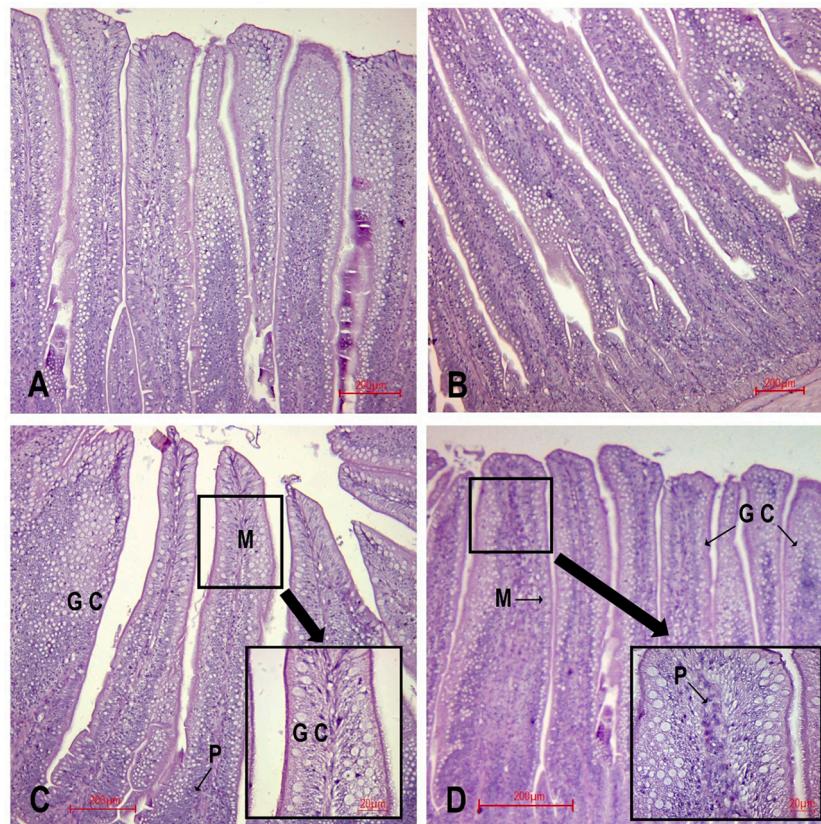


Fig. 1. A gut histological section in broilers fed S, in diet, during 15 and 21 days. (A): C15, (B): C21; (C): T15, (D): T21 (10 X). In C it is shown increased Goblet cells (G) and mucus layer (M) well developed and some Plasmatic cells (P). In D it is shown increased G, M, and P near apical border villi.

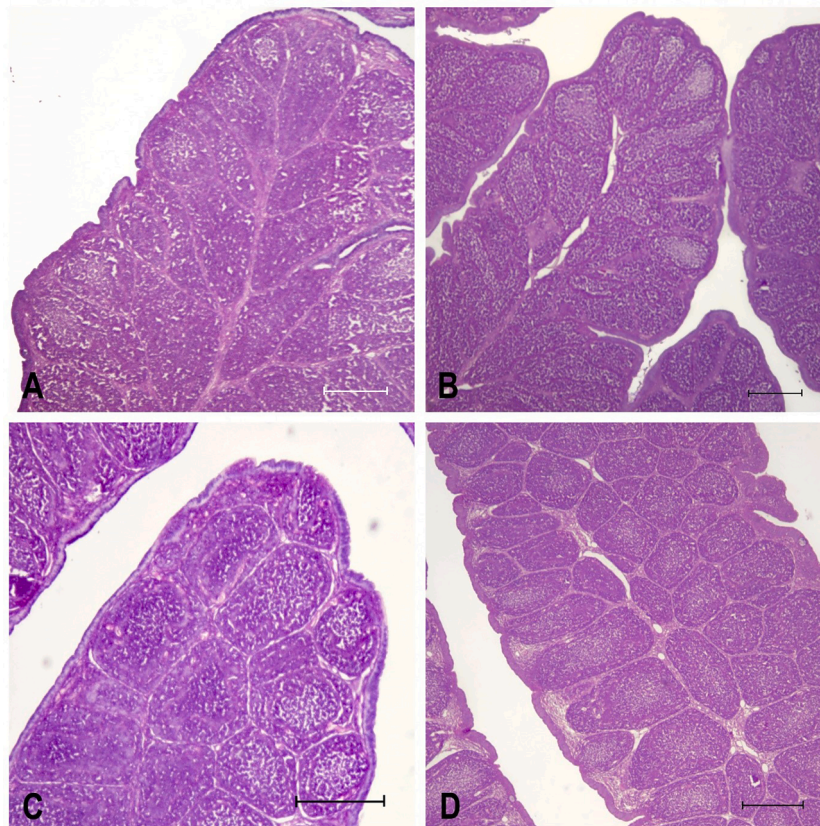


Fig. 2. Fabricius bursa histological section in broilers fed Stevia mash (1 %) during 15 and 21 days. (A): C15, (B): C21; (C): T15, (D): T21 (10 X). In C and D it is shown more follicular develop than A and B. Bar=200 μ .

microbial diversity and evenness, whereas the Simpson index measures the dominance of particular microbial taxa in the microbiome. The Shannon index was similar in the control groups at 15 and 21 days of age. However, at 15 days, the Shannon index decreased to 2.4 in the T15 group, and increased to 3 in the T21 group. The same tendency was observed for Simpson index.

4. Discussion

In modern avian production, characterized by intensive genetic selection, effective management practices, optimal nutrition, and robust health measures are essential. Furthermore, poultry producers are confronted with the challenge of meeting consumer demands for poultry products free from antibiotic residues while ensuring their safety. Concurrently, the rising need for sustainable agroecological models has emerged to preserve the environment aligning with “one health” concept where the interconnectedness of humans, animals, and the environment influences each other. As a result, avian nutrition research is now focused on exploring natural additives that can serve as alternatives to antibiotics as growth promoters (Peralta et al., 2018b; Destoumieux-Garzón et al., 2018; Mackenzie & Jeggo, 2019). Among natural additives, S is a phytobiotic compound with a good perspective to achieve this purpose, although the subjacent mechanism associated with the positive effects on performance productivity is not known yet. Performance productivity is the direct reflection of the functionality of the microbiota-brain-gut axis. Therefore, any additive that modifies this axis mainly when it is developing (15-21 days) will affect the growth performance. (Celi et al., 2019; Kim and Lillehoj, 2019; Peralta et al., 2019; Paradowska et al., 2022; Tellez-Isaías and Latorre, 2022; Ducatelle et al., 2023)

In this research, no significant changes were observed in the productive variables of broilers at T15 and T21. These findings align with

another study where the addition of SM to the broiler diet (0.5-2 %) did not result in any modifications to productive variables (Vaquero et al., 2022). Unlike our findings, others studies reported an increase in the productive performance of broilers that received SE (0.5-1 % in diet, for 15 days) or SM (0.5-1.5 % on diets for 21 days), although in the last research is important to advertise that the measurement of IC was conducted at 49 days of age (Quesada Figueroa, 2011; Peralta et al., 2020). These different results may be due to SM presentation (leaves or extract) that contained different steviosides/rebaudiosides percentages and/or other bioactive compounds present in SM or ES. In addition, SM compounds can change according to each plant, selected part phyto-genic used (leaf or the entire plant), geographical origin, harvesting season, environmental factors, storage conditions, and processing techniques (Peralta et al., 2018b). Probably, the tendency toward better IC could register in broilers receiving 42-45 days of SM on diets. Further research is necessary to clarify this point.

Sustainably improving health, efficiency, and performance are the main trends in modern poultry farming. One strategy to sustainably increase health and productivity is to understand the crosstalk between the microbiota and brain-gut axis before using different natural additives. Therefore, the concept of gastrointestinal functionality together with gut health can be used to monitor animal health, welfare, and performance (Celi et al., 2019; Tellez-Isaías and Latorre, 2022; Vaquero et al., 2022; Ducatelle et al., 2023).

Gastrointestinal functionality is essential for the intensive growth of broilers, mainly in the first fifteen to twenty-one days old, a period in which the most efficient IC is observed. However, the digestive system, Galt and the microbiota can affect broiler growth in different ways. For example, changes in the gut mucosa can influence nutrient assimilation and modify host metabolism, production/secretion of bioactive substances, and energy production. These changes can be noticed in modifications (positive or negative) in the growth and conversion index

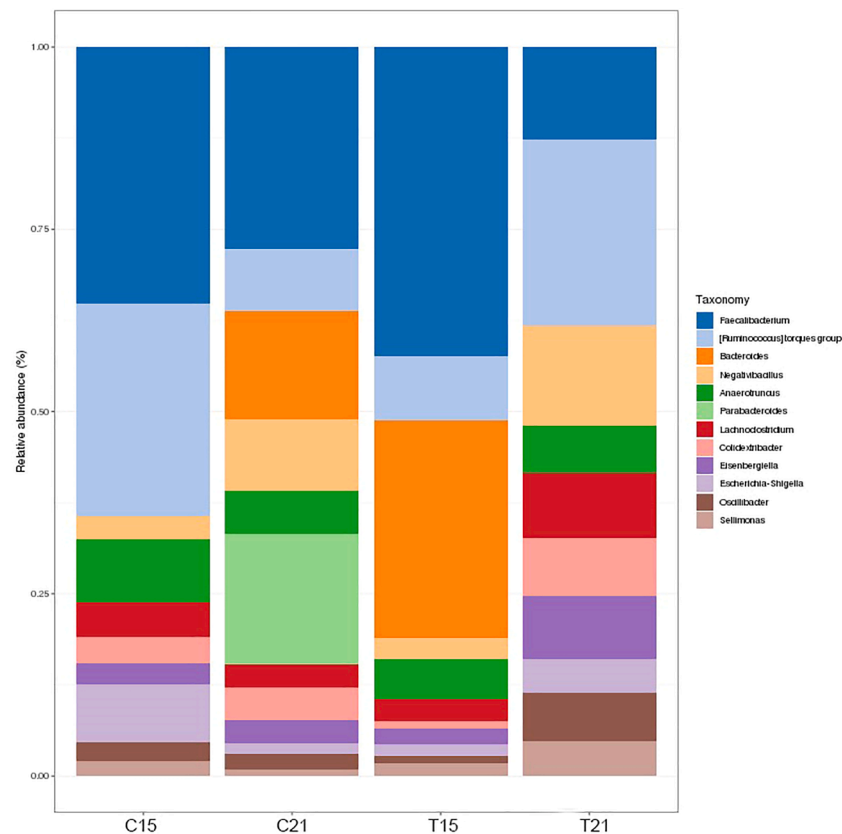


Fig. 3. Distribution of main bacterial taxonomic groups (dominant genera) based on high-throughput sequencing of 16S rRNA gene of the cecal community of broilers fed Stevia mash (1 %) during 15 and 21 days. C: broilers fed control diet. T: broilers fed diet with Stevia mash (1 %). The analysis was performed using the Web-Based Metagenomics Platform SHAMAN.

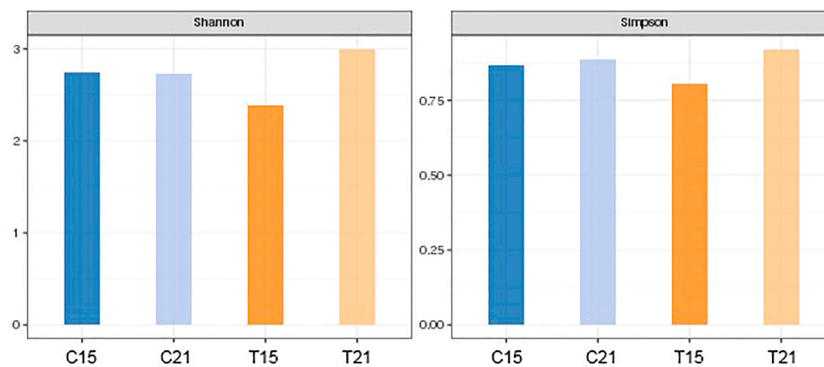


Fig. 4. Diversity analysis of the cecal community of broilers fed Stevia mash (1 %) during 15 and 21 days. C: broilers fed control diet. T: broilers fed diet with Stevia mash (1 %). The analysis was performed using the Web-Based Metagenomics Platform SHAMAN.

(Peralta et al., 2019; Vaquero et al., 2022).

Inside the gut, the intestinal epithelium is constantly exposed to microbiota and antigens; therefore, it is very important for broilers to acquire immunity as soon as possible. In addition, the intestinal immune system has two tasks: controlling pathogenic invaders by immune response and establishing tolerance towards microbiota and antigens contained in feed (Ducatelle et al., 2023). The microbiota interacts with enterocytes, the mucus layer, the gut, and the gut nervous system, affecting gut composition and function (Chen et al., 2015; Celi et al., 2019; Tellez-Isaias and Latorre, 2022; Vaquero et al., 2022). Inside crypts, new enterocytes are formed, which move up the villus while maturing and expressing different receptors and biological signals. Therefore, shorter or elongated villi indicate smaller or larger

absorption areas, respectively. Furthermore, the diminished multiplication of enterocytes in crypts means that it is impossible to compensate for cell loss in the villi. Then, VH/CD is commonly used as an indicator of gastrointestinal functionality. A decrease in VH/CD or an increase in CD is an indicator of a greater need for cell proliferation to maintain gut health (Vaquero et al., 2022; Tellez-Isaias and Latorre, 2022; Ducatelle et al., 2023).

The increased VH/CD ratio in both SM groups indicates that SM generates an increased absorption area, which can be associated with better nutrient assimilation. These results are in agreement with other studies, where noticed an increase in the VH/CD ratio in broilers that received SM (0.5-2 % during 15 days or 0.5-1.5 % during 21 days but measured at 49 days of age) (Quesada Figueroa, 2011; Vaquero et al.,

2022).

In the villi, mucus is produced by the goblet cells, both constitute the first gastrointestinal barrier against enteric pathogen penetration. The IgA is the main immunoglobulin of humoral immunity inside mucosal tissue and contributes to the regulation of the ecological balance between the microbiota, gut, and GALT (Peralta et al., 2017; Kim and Lillehoj, 2019; Vaquero et al., 2022; Ducatelle et al., 2023). Moreover, villi contain epithelial cells and intraepithelial lymphocytes, both constitute the second gastrointestinal barrier.

The mucus layer allows the accommodation of IgA and microbiota as well as the fluid exchange of nutrients. This indicates that an increase in height, both in the inner and external mucus layer, increases gut protection, mainly in the first 15–21 days of age when the GALT is still developing. (Peralta et al., 2016; Kim and Lillehoj, 2019; Kogut, 2019; Tellez-Isaías and Latorre, 2022).

The presence of plasmatic cells, IgA producers, observed near the apical border villi in T21 broilers, suggests that these plasmatic cells could be secreting IgA in the mucus layer earlier than in C21 broilers. Thus, the T21 broilers would acquire the protection against pathogens before the C21 broilers group. These results correlate with the early development of Fabricius Bursa, a primary B cell-producing immune organ.

The microbiome is an essential component of the gastrointestinal tract of animals, including poultry, and plays a crucial role in animal health and productivity. Recent studies have reported the beneficial effects of dietary additives with natural sweeteners such as SM on the gut microbiome of animals. In particular, the cecal microbiome of broilers has been shown to be modulated by SM addition, with changes observed at different ages in the chicken (Kasti et al., 2022). In this study, the pooling of cecal microbiome samples prior to DNA amplification enabled the assessment of community diversity. This approach is widely recognized as a valid measure in population-level association research studies (Ray et al., 2019). The field of metagenomics has rapidly advanced in recent years with the development of new tools and techniques for analysing complex microbial communities. One such tool is the web server SHAMAN, which provides a user-friendly interface for metataxonomic analysis of raw reads from next-generation sequencing. The use of this tool allowed for the efficient processing of a large number of amplicons, resulting in a high-quality dataset for downstream analysis. The identification of 235 OTUs highlighted the complexity of the microbial community under study and provided valuable insights into the diversity of this ecosystem.

The reduction in the relative abundance of *Faecalibacterium* genus observed with the addition of SM at 21 days of age may have implications for gut health and immune function in broilers. It is possible that *Ruminococcus torques* group could replace *Faecalibacterium* in the production of short-chain fatty acids within the broilers group that received SM during the 21-day period. Modulation of the abundance of these genera by the SM addition could potentially impact fiber digestion and overall gut health in broilers, as we noticed on VH/CD ratio. Further research, possibly involving the administration of SM until 42 days old, is needed to elucidate this point. Members of the genus *Bacteroides* are also important constituents of the cecal microbiome whose abundance was modulated by the addition of SM to the diet. Species of *Bacteroides* have been linked to various functions including immune modulation and carbohydrate metabolism, which play crucial roles in the breakdown of complex carbohydrates in the gut. In animals (rats and mice) and humans, it was observed that *Bacteroides*, a specific group of microorganisms present in the intestine, play a crucial role in the hydrolysis of steviol glycosides, such as rebaudioside A and stevioside (Kasti et al., 2022). These compounds are converted into steviol through the microbial transformation process. Subsequently, steviol is transported through the bloodstream to the liver, where it undergoes conjugation. Finally, the conjugated steviol is eliminated from the body through urine. Specific changes in *Bacteroides* abundance with SM addition at the first 15 days might influence the efficiency of nutrient digestion and

absorption in broilers, but these functions could also be associated with another microbial group.

SM addition had a notable impact on the cecal microbiome of broilers, as evidenced by the increased relative abundance of *Negativibacillus*, *Lachnoclostridium*, *Eisenbergiella*, *Colidextribacter*, *Oscillibacter*, and *Sellimonas*. Although the exact role of the genus *Negativibacillus* in the gut microbiome is not well understood, its presence has been associated with beneficial effects on gut health and function (Amit-Romach et al., 2012). The importance of this genus in the broiler gut is unclear; however, its increased abundance with SM addition warrants further investigation. *Eisenbergiella* produces short-chain fatty acids (SCFAs) with anti-inflammatory properties and can improve gut health (McKenna et al., 2020). *Colidextribacter* is a relatively unstudied genus, but some species have been associated with the production of butyrate, a short-chain fatty acid (SCFA) that has been linked to improved gut health in broilers (Lei et al., 2022).

Lachnoclostridium species, belonging to the family Lachnospiraceae, possess the capability to degrade a diverse range of indigestible polysaccharides and ferment dietary fiber for the host. This fermentation process results in the production of beneficial substances like butyric and acetic acid (Lei et al., 2022). Elevated body weight was found to be associated with a high abundance of *Lachnoclostridium* (Lei et al., 2022). Several studies have demonstrated that as body weight increased, there was a corresponding increase in the relative abundance of *Lachnoclostridium* in the cecum (Jacquier et al., 2019). *Oscillibacter* and *Sellimonas* are also important members of the gut microbiome associated with the production of SCFAs and have been shown to have beneficial effects on gut health (Kim et al., 2020; Farkas et al., 2022). The potential benefits of these genera in the chicken gut and their specific roles in response to SM addition require further investigation.

Escherichia-Shigella is a group of Gram-negative bacteria commonly found in the intestinal tract of chickens. Although some strains of *Escherichia-Shigella* are beneficial, others can cause disease and negatively impact animal health. Therefore, understanding the effect of dietary interventions, like SM, on the relative abundance of *Escherichia-Shigella* is essential for optimizing animal production and health. The reduction in the relative abundance of *Escherichia-Shigella* in the treatment group at 15 days of age could be related to the prebiotic effect of SM, which has been reported to stimulate the growth of beneficial bacteria (Maki et al., 2019). The reduction in the relative abundance of *Escherichia-Shigella* may also be related to the shift in the bacterial populations caused by SM addition. *Escherichia-Shigella* has been associated with various diseases, including avian colibacillosis and salmonellosis, which can negatively affect animal health and production (Kabir, 2010). Therefore, a reduction in the relative abundance of *Escherichia-Shigella* in the treatment group could have positive implications on animal health and production.

Simpson and Shannon's indexes suggested that both diversity and evenness of the cecal microbiome were affected by dietary intervention at 15 and 21 days of age. The Simpson index measures the dominance of particular microbial taxa in the microbiome. A decrease in the Simpson index suggests that the dominance of particular taxa is reduced resulting in a more even distribution of microbial species. The prebiotic effects of SM could be responsible for this Simpson index decrease, as has been reported in previous studies. Alternatively, it could be related to changes in the abundance of certain bacterial taxa, such as those affected by SM addition in a previous analysis. Overall, the results obtained from both indexes support the idea that SM addition has a significant impact on cecal microbiome diversity and evenness in the chicken population at 15 and 21 days of age. The similar values of the Shannon index at 15 and 21 days of age might suggest that the cecal microbiome of the chickens is not affected by age. However, at 15 days of age, the decrease in the Shannon index in the T15 group and the increase in the T21 group indicate that the diversity and evenness of the cecal microbiome were affected by dietary intervention. The decrease in the Shannon index at T15 could be related to the natural shift in bacterial populations that

occurs as the chicken gut matures. In contrast, the increase in the Shannon index at T21 could be associated with the prebiotic effect of SM, which has been reported to stimulate the growth of beneficial bacteria (Maki et al., 2019). Thus, the increase in the Shannon index could be the consequence of the shift in bacterial populations caused by SM addition. An increase in microbial diversity could have positive implications on animal health and production. Our results suggest that SM might have a prebiotic effect on the cecal microbiome of broilers by promoting the growth of beneficial bacteria and increasing microbial diversity. It was reported that dietary addition with a phytobiotic mixture increased alpha diversity in the cecal microbiome of broilers, as measured by using the Shannon index (Rodrigues et al., 2020). Similarly, another study found that different phytobiotic mixture led to an increase in alpha diversity, as measured by both the Shannon and Simpson indexes (Pourabedin and Zao, 2015).

The cecal microbiome of broilers plays a vital role in nutrient digestion and absorption, immune functions, and overall health. This study has shown that the addition of SM in the broilers diets affects the composition of the cecal microbiome, with some bacterial genera being affected more than others. Addition of SM to the diet modulates the cecal microbiome of chickens, as evidenced by the changes in the relative abundance of several important bacterial genera, including *Faecalibacterium*, *Bacteroides*, *Ruminococcus* torque group, *Negativibacillus*, *Lachnoclostridium*, *Eisenbergiella*, *Colidextribacter*, *Oscillibacter*, and *Sellimonas*.

Furthermore, *S. rebaudiana*, the plant source of steviol glycosides, contain inulin and fructans. These functional food components have demonstrated beneficial effects on human health (Peteliuk et al., 2021). However, their influence on broiler chickens is still not well understood. The fermentation capacity of fructans as a substrate for the microbiota is dependent on specific strains. Fructans derived from *S. rebaudiana*, particularly those with a polymerization degree less than six (indicating carbohydrates with different chain lengths), have been shown to enhance the growth of select microbial strains, such as *Bifidobacteria* and *Lactobacilli*, which are known to be important for maintaining optimal bowel function (Kasti et al., 2022).

In the study conducted by Kim et al. (2020), the impact of dietary SM addition on the cecal microbiome of broiler chickens was examined. Their findings revealed significant alterations in the composition of the cecal microbiome. Notably, there was an increase in the abundance of beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium*, alongside a reduction in the abundance of potentially harmful bacteria, including *Escherichia coli*. These findings are consistent with those of previous studies that have demonstrated the beneficial effects of SM on the gut microbiome. For example, a study conducted by Wang et al., 2018 found that SM addition increased the abundance of beneficial bacteria, improved gut barrier function, and reduced inflammation in mice.

Our analysis was limited to bacterial microorganisms and worked with a relatively modest sample size. However, it is worth noting that the chicken population exhibited low heterogeneity, attributed to their uniform age, shared origin, and consistent husbandry practices. This allowed the implementation of a sample pooling approach. Pooling may not be a good strategy when the heterogeneity of microbiome samples is high. The changes in the gut microbiome could have beneficial effects on chicken health and productivity. The results presented here suggest that SM has a prebiotic effect on the cecal microbiome of broilers by promoting the growth of beneficial bacteria and reducing the abundance of potentially harmful bacteria; however, its impact could be time-dependent.

5. Conclusion

In summary, SM (1 %) added to the diets produces early immunologic maturity on Fabricius Bursa, increases intestinal functionality, and modifies the cecal microbiome of broilers both at 15 and 21 days.

Chickens were affected by SM addition in a dependent manner on the

chicken age.

Although some changes may have potential implications for gut health and immune function, further research is needed to fully understand the mechanisms by which stevia modulates the gut microbiome of chickens and to determine the optimal dosage and duration of stevia supplementation.

The precise mechanism by which SM impacts the gut microbiome is not yet fully understood. Nonetheless, the phytochemical attributes of SM might also contribute. Phytobiotics encompass non-digestible dietary elements that selectively promote the growth and function of beneficial gut bacteria.

Our results indicate that incorporating SM into the diet could substantially influence the cecal microbiome of broiler chickens, manifesting diverse effects across different age groups. However, further research is required to fully understand the mechanisms underlying these changes and their potential consequences on the Microbiota-brain-gut axis as well as in the broiler health and productivity

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Ethical Statement

We declare that all the experimental procedures applied in this study were reviewed and approved by the by the National University of Rio Cuarto (UNRC) Ethics Committee and conducted in accordance with the Guidelines for Experimental Animals (Olfert, et al., 1993).

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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References

- Amit-Romach, E., Sklan, D., & Uni, Z. (2012). Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. *Poultry Science*, 91(11), 2820–2832. <https://doi.org/10.1093/ps/83.7.1093>
- Atteh, J. O., Onagbesan, O. M., Tona, K., Decuypere, E., Geuns, J. M., & Buyse, J. (2008). Evaluation of supplementary stevia (*Stevia rebaudiana*, Bertoni) leaves and stevioside in broiler diets: effects on feed intake, nutrient metabolism, blood parameters, and growth performance. *Journal of Animal Physiology and Animal Nutrition*, 92(6), 640–649. <https://doi.org/10.1111/j.1439-0396.2007.00760.x>
- Atteh, J. O., Onagbesan, O. M., Tona, K., Decuypere, E., Geuns, J., & Buyse, J. (2011). Potential use of *Stevia rebaudiana* in animal feeds. *Archivos de Zootecnia*, 60(229), 133–136.
- Aviagen Group. Broiler Ross 308: Objetivos de rendimiento. 2012. Retrieved from http://www.es.aviagen.com/assets/Tech_Center/BB_Foreign_Language_Docs/Aviagen-Ross.
- Celi, P., Veerilac, V., Perez Calvo, E., Schmeisser, J., & Klünter, A. (2019). Biomarkers of gastrointestinal functionality in animal nutrition and health. *Animal Feed Science and Technology*, 250, 9–31. <https://doi.org/10.1016/j.anifeedsci.2018.07.012>
- Chen, J., Tellez, G., Richards, J., & Escobar, J. (2015). Identification of potential biomarkers for gut barrier failure in broiler chickens. *Frontiers in Veterinary Science*, 2, 14–24. <https://doi.org/10.3389/fvets.2015.00014>
- Christaki, E., Bonos, E., Giannenas, I., Karatzia, M. A., & Florou-Paneri, P. C. (2013). *Stevia rebaudiana* as a novel source of food additives. *Journal Food Nutrition Research*, 52(4), 195–202.
- Destoumieux-Garzón, D., Mavingui, P., Boetsch, G., Boissier, J., Darriet, F., Duboz, P., Fritsch, C., Giraudoux, P., Le Roux, F., Morand, S., Paillard, C., Pontier, D., Sueur, C., & Voituron, Y. (2018). The one health concept: 10 years old and a long road ahead. *Frontiers in Veterinary Science*, 5, 14. <https://doi.org/10.3389/fvets.2018.00014>. eCollection 2018.
- Di Rienzo J.A.; Casanoves F.; Balzarini M.G.; Gonzalez L.; Tablada M.; Robledo C.W. InfoStat versión 2016. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. URL <http://www.infostat.com.ar>.

- Ducatel, R., Goosens, E., Eeckhaut, V., & Van Immerseel, P. (2023). Poultry gut health and beyond. *Animal Nutrition*, 13, 240–248. <https://doi.org/10.1016/j.aninu.2023.03.005>. eCollection 2023 Jun.
- Farkas, V., Csitári, G., Menyhart, L., Such, N., Pál, L., Husveth, F., Rawash, M. A., Mezölaki, Á., & Dublec, K. (2022). Microbiota composition of mucosa and interactions between the microbes of the different gut segments could be a factor to modulate the growth rate of broiler chickens. *Animals*, 12, 1296. <https://doi.org/10.3390/ani12101296>
- Jacquier, V., Nelson, A., Jjali, M., Rhayat, L., Brinch, K. S., & Devillard, E. (2019). *Bacillus subtilis* 29784 induces a shift in broiler gut microbiome toward butyrate-producing bacteria and improves intestinal histomorphology and animal performance. *Poultry Science*, 98, 2548–2554. <https://doi.org/10.3382/ps/pey602>
- Kabir, S. M. L. (2010). Avian Colibacillosis and Salmonellosis: A closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. *Journal Environment Reseach on Public Health*, 7, 89–114. <https://doi.org/10.3390/ijerph7010089>
- Kasti, A. N., Nikolaki, M. D., Synodinou, K. D., Katsas, K. N., Petsis, K., Lambreinou, S., Pyrrous, I. A., & Triantafyllou, K. (2022). The effects of Stevia consumption on gut bacteria: friend or foe? *Microorganism*, 10, 744. <https://doi.org/10.3390/microorganism10040744>
- Kim, W., & Lillehoj, H. (2019). Immunity, immunomodulation, and antibiotic alternatives to maximize the genetic potential of poultry for growth and disease response. *Animal Food and Science Technology*, 250, 41–50. <https://doi.org/10.1016/j.anifeedsci.2018.09.016>
- Kim, J. W., Choi, H. C., Park, J. W., Lee, S. W., & Kim, K. H. (2020). Effects of dietary supplementation with *Stevia rebaudiana* on the cecal microbiome of broiler chickens. *Poultry Science*, 99(11), 5377–5385.
- Kogut, M. (2019). The effect of microbiome modulation on the intestinal health in poultry. *Animal Feed Science and Technology*, 250, 32–40. <https://doi.org/10.1016/j.anifeedsci.2018.10.008>
- Korver, D. (2022). Antibiotic use impact in poultry immune system and food safety. In Symposium: One Health and antibiotic-free poultry production: Actual global challenges. In *Proceedings of the Conference of Poultry Science Association -3er. LatinAmerican Conference. Foz de Iguazu, Brasil*.
- Lei, J., Dong, Y., Hou, Q., He, Y., Lai, Y., Liao, C., Kawamura, Y., Li, J., & Zhang, B. (2022). Intestinal microbiota regulate certain meat quality parameters in chicken. *Frontiers Nutrition*, 9, Article 747705. <https://doi.org/10.3389/fnut.2022.747705>
- McKenna, A., Ijaz, U. Z., Kelly, C., Linton, M., Sloan, W. T., Green, B. D., Lavery, U., Dorrell, N., Wren, B. W., Richmond, A., Corcionivoschi, N., & Gundogdu, O. (2020). Impact of industrial production system parameters on chicken microbiomes: Mechanisms to improve performance and reduce *Campylobacter*. *Microbiome*, 8(1), 128. <https://doi.org/10.1186/s40168-020-00908-8>
- Mackenzie, J., & Jeggo, M. (2019). The one health approach-why is it so important? *Tropical Medicine Infection Diseases*, 4, 88. <https://doi.org/10.3390/tropicalmed4020088>
- Maki, J. J., Klima, C. L., Sylte, M. J., & Looft, T. (2019). The microbial pecking order: Utilization of intestinal microbiota for poultry health. *Microorganisms*, 7, 376. <https://doi.org/10.3390/microorganisms7100376>
- Mehdi, Y., Letourneau-Montminy, M. P., Gaucher, M. L., Chorfi, L., Suresh, G., Rouissi, T., Kaur Brar, S., Cot, C., Avalos Ramirez, A., & Godbout, S. (2018). Use of antibiotics in broiler production: Global impacts and alternatives. *Animal Nutrition*, 4, 170–178. <https://doi.org/10.1016/j.aninu.2018.03.002>. Epub 2018 Apr 3.
- Olfert, E. D., Cross, B. M., & McWilliam, A. A. (1993). *Guide to the Care and Use of Experimental Animals* (2nd Edition). Ottawa: Canadian Council on Animal Care.
- Paradowska, M., Dunislawski, A., Siwek, M., & Slawinska, A. (2022). Avian cell culture models to study immunomodulatory properties of bioactive products. *Animals*, 12, 670. <https://doi.org/10.3390/ani12050670>
- Peralta, M. F., Danelli, M. G. M., & Vivas, A. B. (2016). Rediscovering the importance of mucosal immune system (MIS) in poultry. *Academia Journal of Biotechnology*, 4(3), 91–95. <https://doi.org/10.5413/ajb.2015.0238>
- Peralta, M. F., Magnoli, A., Alustiza, F., Nilson, A., Miazzo, R., & Vivas, A. (2017). Gut-associated lymphoid tissue: A key tissue inside the mucosal immune system of hens immunized with *Escherichia coli* F4. *Frontiers of Immunology*, 8, 568. <https://doi.org/10.3389/fimmu.2017.0568>
- Peralta, M. F., Nilson, A., Grosso, V., Soltermann, A., & Miazzo, R. D. (2018). Stevia (*Stevia rebaudiana Bertoni*): Un nuevo aditivo natural en avicultura? *Revista de Ciencias Veterinarias*, 36(1), 7–19. <https://doi.org/10.15359/rcv.36-1.1>
- Peralta, M. F., Nilson, A., & Miazzo, R. D. (2018). Getting better intestinal health through the addition of yeast (*Saccharomyces cerevisiae*) combined with threonine in broilers diets. *Applied Science and Immunology Research*, 2(2), 49–62. <https://doi.org/10.22158/asir.v2np49>
- Peralta, M. F., Nilson, A. J., & Miazzo, R. D. (2019). Nutrición aviar: alternativas naturales para optimizar la funcionalidad gastrointestinal. *Ab Intus*, 4(2), 103–109.
- Peralta, M. F., Nilson, A., Grosso, V., Senz, A., Soltermann, A., & Miazzo, R. D. (2020). STEVIA (*Stevia rebaudiana Bertoni*) Extract: A natural alternative in broilers nutrition. *Applied Science and Immunology Research*, 4(4), 38–49. <https://doi.org/10.22158/asir.v4n4p38>
- Peteliuk, V., Rybchuk, L., Bayliak, M., Storey, K. B., & Lushchak, O. (2021). Natural sweetener Stevia rebaudiana: Functionalities, health benefits and potential risks. *EXCLI Journal*, 20, 1412–1430. <https://doi.org/10.17179/excli2021-4211>
- Pourabedin, M., & Zhao, X. (2015). Prebiotics and gut microbiota in chickens. *FEMS Microbiology Letters*, 362(15), fnv122. <https://doi.org/10.1093/femsle/fnv122>
- Quesada-Figueroa, J. (2011). Utilización de tres niveles de *Stevia rebaudiana* en alimentación de broilers y su influencia en flora y desarrollo intestinal. Tesis of grade. Salgolqui, Ecuador. 102 pag.
- Raheem, D., Soltermann, A. T., Tamiozzo, L. V., Cogo, A., Favén, L., Punam, N. J., Sarmiento, C. R., Rainosalo, E., Picco, F., Morla, F., Nilson, A., & Stammler-Gossmann. (2022). Partnership for international development: Finland–Argentina conference on circular economy and bioeconomy with emphasis on food sovereignty and sustainability. *Internacional Journal Environmental Reseach Public Health*, 19, 1773. <https://doi.org/10.3390/ijerph19031773>
- Ray, K. J., Cotter, S. Y., Arzika, A. M., Kim, J., Boubacar, N., Zhou, Z., Zhong, L., Porco, T. C., Keenan, J. D., Lietman, T. M., & Doan, T. (2019). High-throughput sequencing of pooled samples to determine community-level microbiome diversity. *Annals of Epidemiology*, 39, 63–68. <https://doi.org/10.1016/j.annepidem.2019.09.002>
- Rodrigues, D. R., Briggs, W., Duff, A., Chasser, K., Murugesan, R., Pender, C., Ramirez, S., Valenzuela, L., & Bielke, L. R. (2020). Comparative effectiveness of probiotic-based formulations on cecal microbiota modulation in broilers. *PLoS ONE*, 15(5), Article e0225871. <https://doi.org/10.1371/journal.pone.0225871>
- Rostagno, H.S. (2017). Chap. 2: Exigencias nutricionais das Aves. In *Tabelas brasileiras para Aves e suínos*- Univ. Federal deViçosa. Viçosa-Brasil. 4th ed.: 259-398.
- Taha-Abdelaziz, K., Hodgins, D., Laamers, A., Alkie, T., & Sharif, F. (2018). Effects of early feeding and dietary interventions on development of lymphoid organs and immune competence in neonatal chickens: A review. *Veterinary Immunology Immunopathology*, 21, 1–11. <https://doi.org/10.1016/j.vetimm.2018.05.001>
- Tellez-Isaías, G; Latorre, J. (2022). Editorial: *Alternatives to antimicrobial growth promoters and their impact in gut microbiota, health and disease*. Vol 2. Lausanne: Frontiers Media SA. 10.3389/fvets.2022.857583. eCollection 2022.
- Vaquero, M., Nilson, A., Grosso, V., Soltermann, A., Miazzo, R. D., & Peralta, M. F. (2022). Natural additives in broilers: Stevia (*Stevia rebaudiana Bertoni*) as an innovator growth promoter. *E-Cronical Veterinary Science*, 7(5), 1–9.
- Volant, S., Lechat, P., Woringer, P., Motreff, L., Campagne, P., Malabat, C., Kennedy, S., & Ghozlane, A. (2020). SHAMAN: A user-friendly website for metataxonomic analysis from raw reads to statistical analysis. *BMC Bioinformatic*, 21(1), 345. <https://doi.org/10.21203/rs.2.23213/v1>
- Wang, Q. P., Browman, D., Herzog, H., & Neely, G. G. (2018). Non-nutritive sweeteners possess a bacteriostatic effect and alter gut microbiota in mice. *PLoS One*, 13(7), Article e0199080. <https://doi.org/10.1371/journal.pone.0199080>
- Xu, Q., Liu, M., Chao, X., Zhang, C., Yang, H., Chen, J., & Zhou, B. (2023). Stevioside improves antioxidant capacity and intestinal barrier function while attenuating inflammation and apoptosis by regulating the NF- κ B/MAPK pathways in diquat-induced oxidative stress of IPEC-J2 cells. *Antioxidants*, 12, 1070. <https://doi.org/10.3390/antiox12051070>