



## Gut microbiome and morphometry of quails fed diets containing essential oils

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**ABSTRACT:** One of the greatest challenges in poultry production is maintaining intestinal mucosal barrier integrity and gut microbiota balance. Safe alternative antimicrobials that can regulate the microbial community through animal feed have been the subject of research in poultry production. This study evaluated the effect of *Mentha piperita* and *Melaleuca alternifolia* essential oils (EOs) on the gut microbiome and morphometry of broiler quails under normal feeding conditions. The gut microbiome was studied using a completely randomized design consisting of 4 treatments, namely control, bacitracin zinc, and the EOs *M. piperita* and *M. alternifolia*, with 8 repetitions and 7 quails per treatment, totaling 224 quails from 1 to 42 days old. The intestinal contents of the slaughtered quails were collected to evaluate the gut microbiome profile of their digestive tract. Gut morphometry was analyzed using a completely randomized factorial design, with four experimental rations for three intestinal sections (4x3) and five replications. The variables studied were villus surface area and height, crypt depth, villus height to crypt depth ratio (VH:CD), villus-crypt ratio (V:C), villus width to height ratio (VW:H), and height of the intestinal epithelium and musculature. *M. alternifolia* (50 mg/kg of feed) in the diet of broiler quails improved gut morphometry, similar to the results obtained with bacitracin zinc. This EO also altered the gut microbiome of quails and reduced pathogenic bacterial diversity.

**Key words:** *Mentha piperita*, *Melaleuca alternifolia*, microbial diversity, *Coturnix coturnix*.

### Microbioma intestinal e morfometria de codornas alimentadas com dietas contendo óleos essenciais

**RESUMO:** Um dos maiores desafios na produção de aves é manter a integridade da barreira da mucosa intestinal e o equilíbrio da microbiota intestinal. Antimicrobianos alternativos seguros, que podem regular a comunidade microbiana através da alimentação animal, têm sido objeto de pesquisa na produção avícola. O objetivo deste estudo foi avaliar o efeito dos óleos essenciais (OEs) *Mentha piperita* e *Melaleuca alternifolia* no microbioma intestinal e na morfometria de codornas de corte em condições normais de alimentação. O microbioma intestinal foi estudado em delineamento inteiramente casualizado com 4 tratamentos: controle, bacitracina zinco e os OEs *M. piperita* e *M. alternifolia*, com 8 repetições, por tratamento, com sete codornas totalizando 224 aves no período de um a 42 dias. O conteúdo intestinal das codornas abatidas foi coletado para avaliar o perfil do microbioma intestinal de seu trato digestivo. A morfometria intestinal foi estudada usando um delineamento fatorial inteiramente casualizado, consistindo de quatro rações experimentais para três seções intestinais (4x3), com cinco repetições. As variáveis estudadas foram área das vilosidades intestinais, altura das vilosidades intestinais, profundidade da cripta intestinal, relação entre a altura das vilosidades e a profundidade das criptas, relação cripta e vilosidades intestinais, relação largura e altura das vilosidades intestinais, altura do epitélio intestinal e altura da musculatura intestinal. A utilização do OE *M. alternifolia* (50 mg/kg de ração) na dieta de codornas de corte resultou em melhora da morfometria intestinal, semelhante aos resultados obtidos com bacitracina zinco. Além disso, alterou o microbioma intestinal de codornas e reduziu a diversidade bacteriana patogênica.

**Palavras-chave:** diversidade microbiana, *Coturnix coturnix*, *Melaleuca alternifolia*, *Mentha piperita*.

### INTRODUCTION

The gut microbiome is a set of microorganisms that colonize the intestinal mucosa and lumen (BEDANI & ROSSI (2009); THOMAS & GREER (2010)). This microbiome directly affects animal health, performance, immunology and gastrointestinal (GI) tract physiology and performance (CLAVIJO & FLOREZ (2018)). The GI tract of broiler quails (*Coturnix coturnix*) is home to unique microbial communities that play an essential role in their health and well-being. (WILKINSON et al. (2016)).

One of the greatest challenges in poultry production is maintaining intestinal mucosal barrier

integrity and gut microbiota balance. Performance-enhancing antibiotics have been used to help maintain GI tract microbiota stability through competitive exclusion, resulting in better host immunity and performance (BELLONI et al. (2012); LEMOS et al. (2016)). However, restrictions on the use of antimicrobials as performance enhancers prompted a search for alternatives to maintain previously established production patterns (BRENES & ROURA (2010); KOIYAMA et al. (2014)).

Safe alternative antimicrobials that can regulate the microbial community through animal feed have been the subject of research in poultry

production. Performance enhancers based on plant extracts, probiotics, prebiotics, acidifiers, and essential oils (EOs) improve feed efficiency and animal health due to the large number of molecules with medicinal potential PIZZILO et al. (2011); KLASSA et al. (2013); HAJIAGHAPOUR & REZAEIPOUR (2018); REDA et al. (2020); ALAGAWANY et al. (2021).

Among alternative antimicrobials, EOs are the main group of plant-based phytochemical feed additives that favor intestinal function by stimulating digestive secretions, increasing enzyme activity, and reducing pathogen adherence HALL et al. (2021); PRAJAPATI, et al. (2021).

Peppermint (*Mentha piperita*) EO is known to have antimicrobial, anti-inflammatory, antiviral, antihypertensive, antioxidant, antiallergic, biopesticidal and larvicidal properties. According to research, its two main active components, menthol and menthone, improve the intestinal health of poultry MAHENDRAN & RAHMAN (2020); OLUWAFEMI et al. (2020)

Tea tree (*Melaleuca alternifolia*), an EO from the family Myrtaceae, is listed as a phytochemical substance with antimicrobial, antifungal, anthelmintic, antiviral, antitumor, and anti-inflammatory activities NEPOMOCENO & PIETROBON (2020). Moreover, its primary components are terpinen-4-ol and  $\gamma$ -terpinene, which confer its medicinal properties. As a performance-enhancing antimicrobial, this oil can also be used as a poultry diet supplement to increase productivity PUVAČA et al. (2019).

Thus, this study evaluated the effect of *M. piperita* and *M. alternifolia* EOs on the gut microbiome and intestinal morphometry of quails under normal feeding conditions.

## MATERIALS AND METHODS

It was conducted between October and December 2019 at the experimental quail farming facility belonging to the Aviculture Laboratory of the State University of Southwest Bahia (UESB), in Itapetinga, BA, Brazil (15° 09' 07" S, 40° 15' 32" W and altitude of 268 meters).

A total of 224 one-day-old quails were used, 50% of each sex, purchased from Aya Farm in Suzano, São Paulo state (SP). From 1 to 42 days old, the quails were housed in a brooder shed containing 32 experimental cages, under a 24-hour light photoperiod.

### Essential oil-supplemented diets

Quail nutritional requirements vary according to their developmental stage, whereby specific feed may be needed depending on days of life. The feeding program

was divided into two stages, with the first stage from 1 to 21 and the second from 22 to 42 days old.

The diets (Table 1) consisted of ground corn and soybean meal, in line with the nutritional requirements and feed composition recommended by SILVA & COSTA (2009). The metabolizable energy value used as a reference for soybean oil was based on ROSTAGNO et al. (2017).

EOs were added to the feed during the manufacturing process, adapted from DAIRIKI et al. (2013). Soybean oil and EO mixtures were prepared for each kilogram of feed in the experimental treatments, using soybean oil as a vehicle for EO incorporation. The control group received standard quail feed with only soybean oil.

According to DEMINICIS et al. (2021), there are no data on standardized EO doses for quails. As such, doses capable of combatting the main intestinal disease pathogens in poultry farming were used, corresponding to the minimum bactericidal concentration (MBC) determined in vitro via the microdilution method in a previous study conducted by our team. The results were 12.5 and 50 mg of EO per kg of feed for *M. piperita* and *M. alternifolia*, respectively (unpublished data).

At 42 days old, the quails were weighed and one bird with the average weight of each batch was separated for collection, totaling 32 quails slaughtered. The birds received food and water *ad libitum* throughout the experimental period until slaughter by desensitization with cervical dislocation, performed by a trained professional, followed by exsanguination.

### Quail microbiome analysis

The intestinal microbiome was studied using a completely randomized design (CRD), consisting of 4 treatments (control, bacitracin-zinc and EOs *M. piperita* and *M. alternifolia*) with 8 replications, totaling 32 quails from 1 to 42 days old. The intestinal contents of the slaughtered quails were collected to evaluate the microbiome profile of their digestive tract. Each treatment was "pooled" to diagnose the gut microbiome, totaling four samples for microbiome analysis.

New generation sequencing (Neoprosperta Microbiome Technologies, Brazil) of the V3/V4 regions of the 16S ribosomal ribonucleic acid (rRNA) gene was performed CHRISTOFF et al. (2019) and the results recorded in relative abundance as a percentage of sequence reads. A total of 50,000 reads were analyzed per treatment.

Amplification was carried out using primers for the V3-V4 region of the 16S rRNA gene (341

Table 1 - Centesimal composition of experimental diets.\*

Ingredient (%)	-----Initial (1 to 21 days) -----				-----Growth (22 to 42 days) -----			
	T1	T2	T3	T4	T1	T2	T3	T4
Corn	52.85	52.82	52.85	52.85	59.99	59.97	59.97	59.97
Soybean meal	42.00	42.00	42.00	42.00	35.4	35.39	35.39	35.39
MHL – Methionine (84%)	0.5	0.5	0.5	0.5	0.45	0.45	0.45	0.45
Lysine Sulfate (55%)	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
L- Threonine (98%)	0.25	0.25	0.25	0.25	0.00	0.00	0.00	0.00
Calcitic lime	1.02	1.02	1.02	1.02	0.96	0.96	0.96	0.96
Dicalcium phosphate	1.32	1.32	1.32	1.32	0.87	0.87	0.87	0.87
Common Salt	0.4	0.4	0.4	0.4	0.33	0.33	0.33	0.33
Premix**	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Soy oil	1.16	1.16	1.06	1.05	1.5	1.5	1.38	1.36
Oil of <i>M. piperita</i>	-	-	0.096	-	-	-	0.125	-
Oil of <i>M. alternifolia</i>	-	-	-	0.113	-	-	-	0.147
Bacitracin zinc	-	0.03	-	-	-	0.03	-	-
Calculated composition	-----Nutritional levels-----							
Metabolizable energy, kcal/kg	2900	2900	2900	2900	3025	3025	3025	3025
Crude protein, %	23.72	23.72	23.72	23.72	21.34	21.34	21.34	21.34
Crude fiber, %	2.82	2.82	2.82	2.82	2.61	2.61	2.61	2.61
Calcium, %	0.92	0.92	0.92	0.92	0.76	0.76	0.76	0.76
Available Phosphorus, %	0.42	0.42	0.42	0.42	0.33	0.33	0.33	0.33
Lysine dig., %	1.51	1.51	1.51	1.51	1.34	1.34	1.34	1.34
Dig methionine., %	0.77	0.77	0.77	0.77	0.7	0.7	0.7	0.7
Methionine + cystine dig., %	0.73	0.73	0.73	0.73	0.68	0.68	0.68	0.68
Threonine dig., %	1.17	1.17	1.17	1.17	0.84	0.83	0.83	0.83
Valine dig., %	1.15	1.15	1.15	1.15	1.03	1.03	1.03	1.03
Sodium, %	0.17	0.17	0.17	0.17	0.14	0.14	0.14	0.14
Linoleic acid, %	1.22	1.22	1.22	1.22	1.3	1.3	1.3	1.3

\*T1: Basal diet (negative control group); T2: Basal diet and 15% bacitracin zinc; T3: Basal diet and *Mentha piperita* essential oil and T4: Basal diet and *Melaleuca alternifolia* essential oil.

\*\*Guaranteed levels per kg of feed for the initial phase: Vitamin A 9996 I.U.; Vitamin D3 1248 I.U.; Vitamin E 20.16 I.U.; Vitamin K3 3 mg; Vitamin B1 3 mg; Vitamin B2 6 mg; Vitamin B6 4 mg; Vitamin B12 0.01008 mg; Niacin 40000mg; Pantothenic Acid 30 mg; Choline 260 g; Folic Acid 0.48 mg; Methionine 1580 mg; Copper 7.99 mg; Iron 499 mg; Manganese 0.07 mg; Zinc 49.99 mg; Iodine 1.20 mg; Selenium 0.19 mg; Salinomycin 550 mg. Guarantee levels per kg of feed for the final phase: Vitamin A 12000 I.U. Vitamin D3 3600 I.U. Vitamin E 180 U.I/kg; Vitamin K3 3.00 mg; Vitamin B1 3.60 mg; Vitamin B2 9.00 mg; Vitamin B6 6.00 mg; Vitamin B12 0.021 mg; Niacin 600 mg; Pantothenic Acid 22.50 mg; Folic Acid 0.150 mg; Choline 510 mg; Iron 7500 mg; Manganese 105 mg; Zinc 75 mg; Copper 15 mg; Iodine 1.80 mg; Selenium 0.30 mg.

FCCTACGGGRSGCAGCAG (WHIPPS, 2001), and 806R GGACTACHVGGGTWTCTAAT (WANG & QIAN, 2009).

The first polymerase chain reaction (PCR 1) was performed using universal V3/V4 primers containing a partial Illumina sequencing adapter, based on the TruSeq structure adapter (Illumina, USA), which allows a second PCR (PCR 2) with similar indexing sequences to the procedures previously described by CAPORASO et al. (2011).

Two microliters of deoxyribonucleic acid (DNA) from the samples were used in each PCR 1 reaction. The reactions were performed using

Platinum Taq DNA polymerase (Invitrogen, USA) under the following conditions: for PCR 1, 95°C for 5 min, 25 cycles at 95°C for 45 seconds, 55°C for 30 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 2 minutes; and for PCR 2, 95°C for 5 minutes, 10 cycles at 95°C for 45 seconds, 66°C for 30 seconds, 72°C for 45 seconds, and a final extension at 72°C for 2 min. All the PCR reactions were conducted in triplicate and a negative control reaction (NCR) was included in each PCR batch. The final PCR reactions were purified with AMPureXP beads (Beckman Coulter, USA) and the samples pooled in the sequencing libraries for quantification.

The libraries were sequenced using the MiSeq System (Illumina Inc., USA) and V2 kit, with 300 cycles and single-end sequencing.

The DNA concentration of the libraries was estimated via double-stranded DNA (dsDNA) assays using PicoGreen® (Invitrogen, USA), and diluted for precise PCR quantification with the KAPA Library Quantification Kit for Illumina platforms (KAPA Biosystems, MA). The library sets were adjusted to a final concentration of 11.5 (for V2 kits) or 18 pM (for V3 kits) and sequenced in a MiSeq system (Illumina, USA), using the standard Illumina primers provided in the manufacturer's kit. Three hundred single-cycle runs were performed using V2x300, V2x300 Micro, V2x500, or V3x600 sequencing kits (Illumina, USA), always generating 283pb size amplifiers suitable for analysis. Coverage for each sequenced sample was established as 10,000 reads.

All the resulting DNA sequences were passed individually through a quality filter based on the sum of the error probabilities of their bases, allowing a maximum 1% accumulated error. The sequences submitted to the initial procedures that obtained 100% identity were grouped into phylotypes/clusters and used for taxonomic identification, by comparison against a database of accurate 16S rRNA sequences (NeoRef, Neoprospecta Microbiome Technologies, Brazil) CHRISTOFF et al. (2019).

#### Cluster analysis

Cluster analysis was based on a binary matrix representing the presence/absence of operational taxonomic units (OTUs) in each treatment. The similarity matrix was obtained according to the Dice similarity coefficient ( $D_s$ ) between pairs. Clusters were determined by sequentially comparing the patterns and constructing a relatedness dendrogram with 100 bootstrap repetitions reflecting the relative similarities.  $D_s$  calculation and cluster analysis were performed in PAST software HAMMER et al. (2001). The Venn diagrams were constructed manually, considering the intersections of the bacterial species identified by sequencing analysis.

#### Gut morphometric analysis

Gut morphometry was analyzed using a CRD with a 4x3 factorial, as follows: four experimental rations (4 treatments: control, bacitracin zinc, and EOs *M. piperita* and *M. alternifolia*) for three intestinal sections (duodenum, jejunum, and ileum), with five repetitions and only one quail per experimental unit, resulting in 20 slaughtered quails, totaling 60 samples (intestinal fragments).

The small intestine fragments (duodenum, jejunum, and ileum) were collected 3 cm from the gizzard, up to 3 cm before the final portion (cecum), and kept fixed in a 10% buffered formalin solution for 48 hours. After collection, the samples were washed in saline solution, fixed in 10% formaldehyde, then dehydrated in a series of increasing concentrations of alcohols, diaphanized in xylol, and embedded in paraffin.

Five 7µm-thick transverse and semi-serial sections were obtained by microtomy and stained with hematoxylin-eosin using the periodic acid-Schiff (PAS) method. Finally, the specimens were mounted on slides with Entellan resin (rapid mounting medium for microscopy) and covered with coverslips. Histological images of the duodenum, jejunum, and ileum were analyzed under a Leica M10 optical microscope in a semi-automatic computational routine, using Image Pro-Plus 4.5 (IPP4.5) software, as described by REIS et al. (2016).

Villus morphometric data were obtained from the three portions of the small intestine (duodenum, jejunum, and ileum) to determine the villus surface area and height, crypt depth, villus width epithelial and muscle height, and villus-crypt ratio (V:C). Finally, 10 measurements per sample were obtained with Image Pro Plus 4.5 software.

#### Statistical analysis

The microbiota was statistically analyzed using STAMP software PARKS et al. (2014). Differences were considered significant when  $P < 0.05$ .

Analyses were performed to assess the effects of the intestine section treatments (control, bacitracin zinc, *M. piperita*, and *M. alternifolia*) and treatment-section interaction using PROC MIXED" of the Statistical Analysis System (SAS, 2011). When interaction was significant, the adjusted means were regressed for each section, thus obtaining the maximum or minimum levels when the regressions were quadratic. In the event of nonsignificant interactions and significant treatment effect, regressions were applied to the adjusted means and obtained for both sections.

## RESULTS AND DISCUSSION

#### Gut microbiome of broiler quails

A total of 166,123 OTUs were identified across the treatments through comparison with a database of accurate 16S rRNA sequences, distributed into seven phyla, namely Firmicutes (151,489), Actinobacteria (13,172), Verrucomicrobia (974), Proteobacteria (345), Bacterioidetes (107), Euryarchaeota (34), and Fusobacteria

(2). The most abundant OTUs were those belonging to the phylum Firmicutes, with mean relative abundance of 90.05%, followed by Actinobacteria at 9.19%. The remaining OTUs, representing Verrucomicrobia, Proteobacteria, Bacteroidetes, Euryarchaeota, and Fusobacteria, obtained a combined mean relative abundance of 0.76% (Figure 1A).

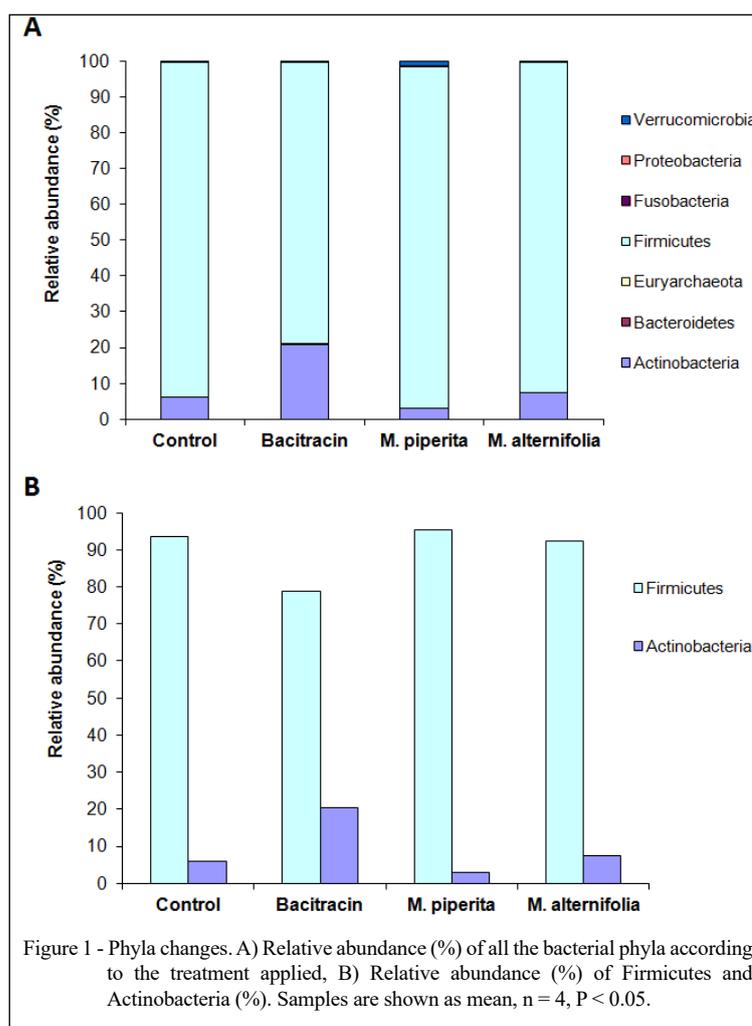
For the two predominant phyla, the proportions of bacteria differed between treatments (Figure 1B). Firmicutes showed greater relative abundance in the control (93.65%), *M. piperita* (95.35%), *M. alternifolia* (92.41%), and bacitracin treatments (78.79%), while Actinobacteria, the second most predominant phylum, obtained 20.54% for bacitracin, greater than that of the control (5.95%), *M. piperita* (2.91%), and *M. alternifolia* treatments (7.35%).

In a study with overweight and obese mice and adult humans, DE SOUZA et al. (2015) identified

a modification in the composition of intestinal microbiota and reported a close relationship between body composition and phyla proportions. According to the authors, a higher Firmicutes/Bacteroidetes ratio is considered a good biomarker for obesity, while an increase in the relative proportion of Bacteroidetes decreases the Firmicutes and Actinobacteria phyla.

In the present study, the Firmicutes/Actinobacteria ratio is high and Bacteroidetes percentages very low (approximately 0.1%). PAIXÃO & CASTRO (2016) reported that the Firmicutes hamper food conversion into energy, thereby compromising weight gain. Notably, the average weight of the quails used here for gut microbiome collection was considered normal for the species at 42 days old in a hot climate (approximately 250 to 280g), as previously reported by DEMINICIS et al. (2022).

These results corroborated those of DU et al. (2020), who studied the gut microbiome of quails



and observed that the Firmicute phylum predominated in the small intestine and was among the five most abundant phyla in the quail intestinal tract. In addition, KÜREKCI et al. (2021) reported that the quail microbiome consists primarily of Firmicutes, Actinobacteria, Bacteroidetes and Proteobacteria.

According to WILKINSON et al. (2016), the primary taxonomic phylum is composed mainly of Firmicutes, Bacteroidetes, and Proteobacteria, with Tenericutes as a novel phylum in quail intestines. It should be noted that, in the present study, the EO treatments did not significantly alter the existing microbial profile when compared to controls, suggesting that these products do not harm the intestinal bacterial community at the tested concentration, thus contributing to gut microbiota variability and balance.

Predicting how microbiome composition affects gut physiology and morphology is challenging JANSSENS et al. (2018), given the limited number of studies characterizing the gut microbiota of broiler quails. As such, the extent to which variations in bacterial relative abundance impact host intestinal immunity has yet to be fully elucidated WILKINSON et al. (2016); DU et al. (2020); BORDA-MOLINA et al. (2020); BROWN et al. (2021).

While general variations in bacteria at genus level were recorded in all the treatments (Figure 2A) when compared to controls, there were notable changes in quails treated with *M. alternifolia*. However, almost all genera were maintained by the treatments, despite changes in relative abundance. By contrast, a reduction in *Lactobacillus* abundance was observed in the bacitracin (29.72%), *M. piperita* (37.98%), and *M. alternifolia* treatments (11.45%) in relation to the control (55.56%) (Figure 2B).

*Lactobacillus* bacteria are considered beneficial to quails because they prevent pathogens from adhering to the intestinal epithelium BORDA-MOLINA et al. (2016). The decline in *Lactobacillus* may be associated with higher intestinal pH or the presence/increase of bacteria of other genera BORDA-MOLINA et al. (2020) and may have enabled greater abundance of *Enterococcus* and *Streptococcus* (lactic acid fermenters) when using *M. piperita* and *M. alternifolia*. By contrast, there was an increase in *Bifidobacterium* species in the bacitracin (19.53%) and *M. alternifolia* treatments (7.20%) in relation to controls and *M. piperita* (1.72 and 2.20%). The heterofermentative bacteria of this genus produce acetic and lactic acid at a molar ratio of 3:2 MACEDO et al. (2008).

According to GONG et al. (2007), the colonizing community tends to be dominated by lactic acid

fermenters. However, *Enterococcus* and *Streptococcus* are gram-positive facultative or obligate anaerobic bacteria with greater difficulty resisting antibiotics PILARSKI & SCHOCKEN-ITURRINO (2010); LOUREIRO et al. (2016), which may explain why these genera stood out.

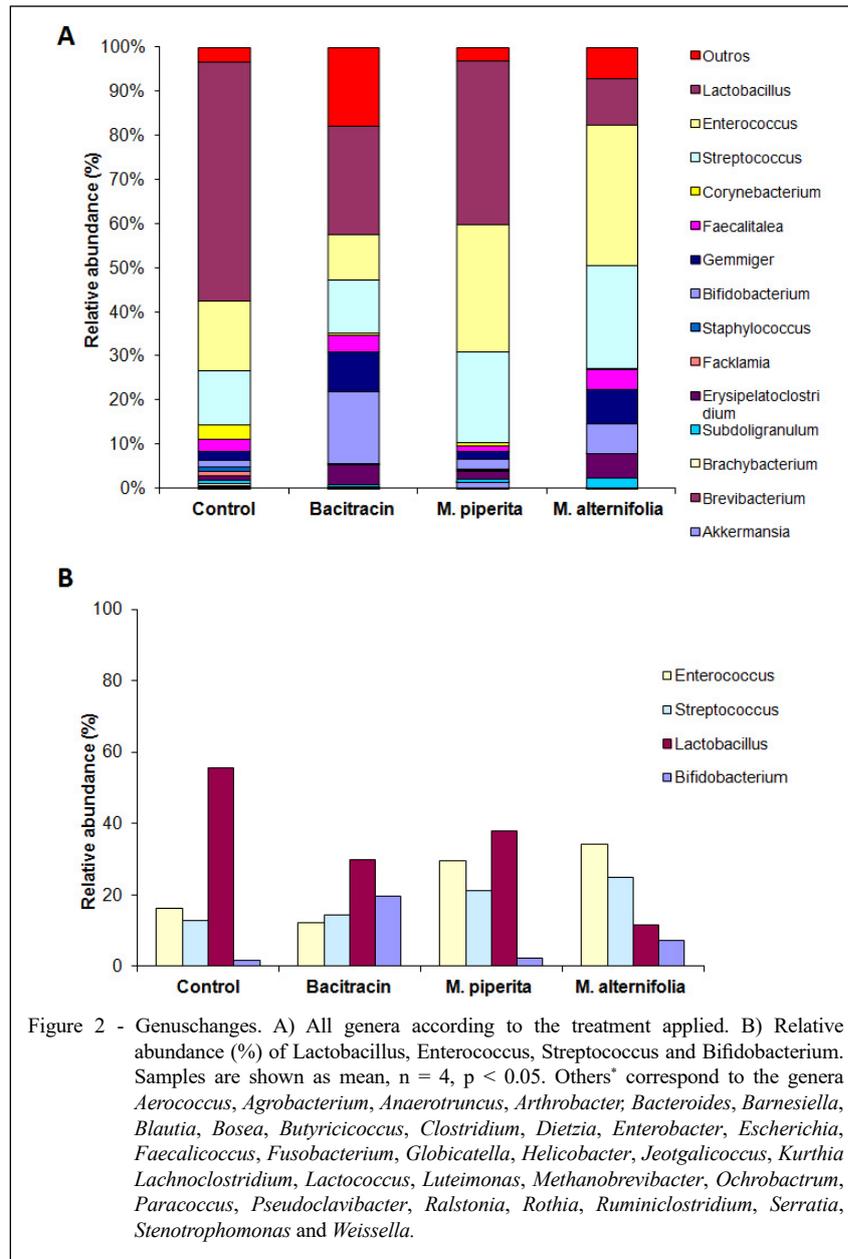
Bacitracin is a potent antibiotic that blocks the transformation of pyrophosphate-bactoprenol to phosphobactoprenol BAPTISTA (2013), an important precursor of cell wall synthesis and a membrane carrier associated with anabolic processes in gram-positive bacteria SCHNEIDER et al. (2009); COSTA et al. (2018). Thus, the imbalance caused by factors internal or external to the host, such as food, antibiotic use, age, and stress, among others, is reflected in the modified gut microbiome. This may lead to an increase/decrease in pathogenic and non-pathogenic bacteria, characterizing dysbiosis, which is typically not positive ZHANG et al. (2015); CARDOSO (2016); ARAÚJO et al. (2019).

General variations of pathogenic species were recorded in all the treatments in relation to the control, but were most evident in quails treated with *M. alternifolia* (Figure 3A). The mean abundance of *Corynebacterium stationis* and *Enterococcus faecalis* declined significantly in all the treatments (2.07 and 2.62%, respectively) when compared to controls, but the relative abundance of *Enterococcus cecorum* increased for bacitracin (84.04%), *M. piperita* (95.28%) and *M. alternifolia* (98.92%) in relation to the control (75.78%) (Figure 3B).

These potential pathogens inhabit the gut microbiota and, depending on their arrangement, can remain there without causing disease because their levels in the host's intestinal lumen are controlled (equilibrium). A disturbance in this balance can trigger the abnormal growth of disease-causing microorganisms MACARI et al. (2014).

*E. cecorum* strains were considered harmless commensals of the GI tract in quails. However, in the last 15 years, pathogenic strains of *E. cecorum* have become a significant cause of morbidity and mortality in broiler matrices and are considered an emerging pathogen in the global poultry industry, whereby strains with greater antimicrobial resistance exhibit increased pathogenicity and share several putative virulence genes DOLKA et al. (2017); JUNG et al. (2018).

As shown in figure 3, in the control group *E. cecorum* encountered low competitiveness from the other types of bacteria present. In regard to the effect of the antimicrobials tested, whether commercial (bacitracin) or alternative (*M. piperita* and *M. alternifolia*), they were notably able to fight

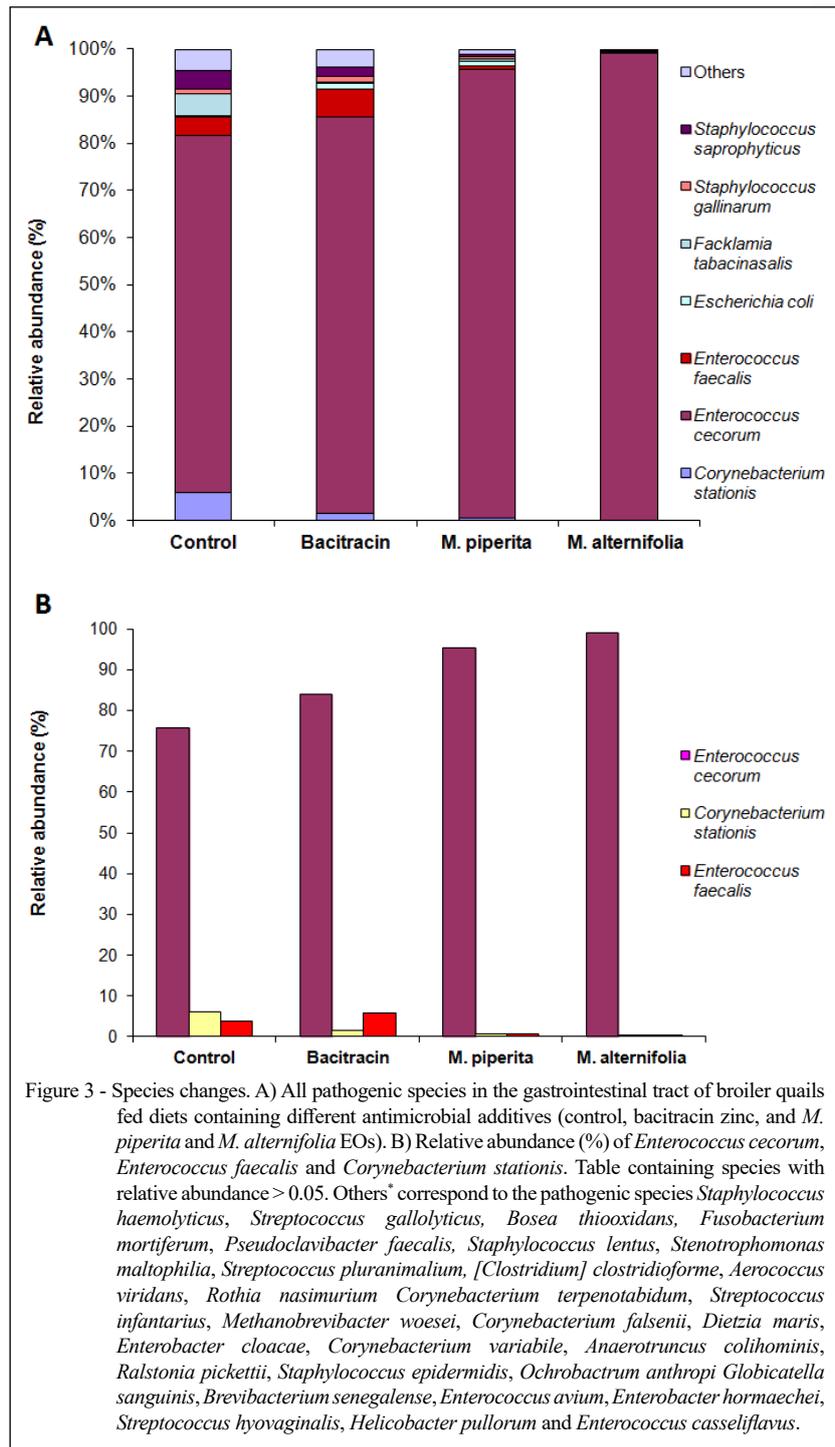


the other bacterial strains present, but ineffective against strains of *E. cecorum*, which may explain their dominance in the intestinal environment by decreasing pathogenic bacterial diversity.

General alterations in species considered non-pathogenic in the literature were observed in all the treatments in relation to controls, especially in quails treated with *M. alternifolia* (Figure 4A).

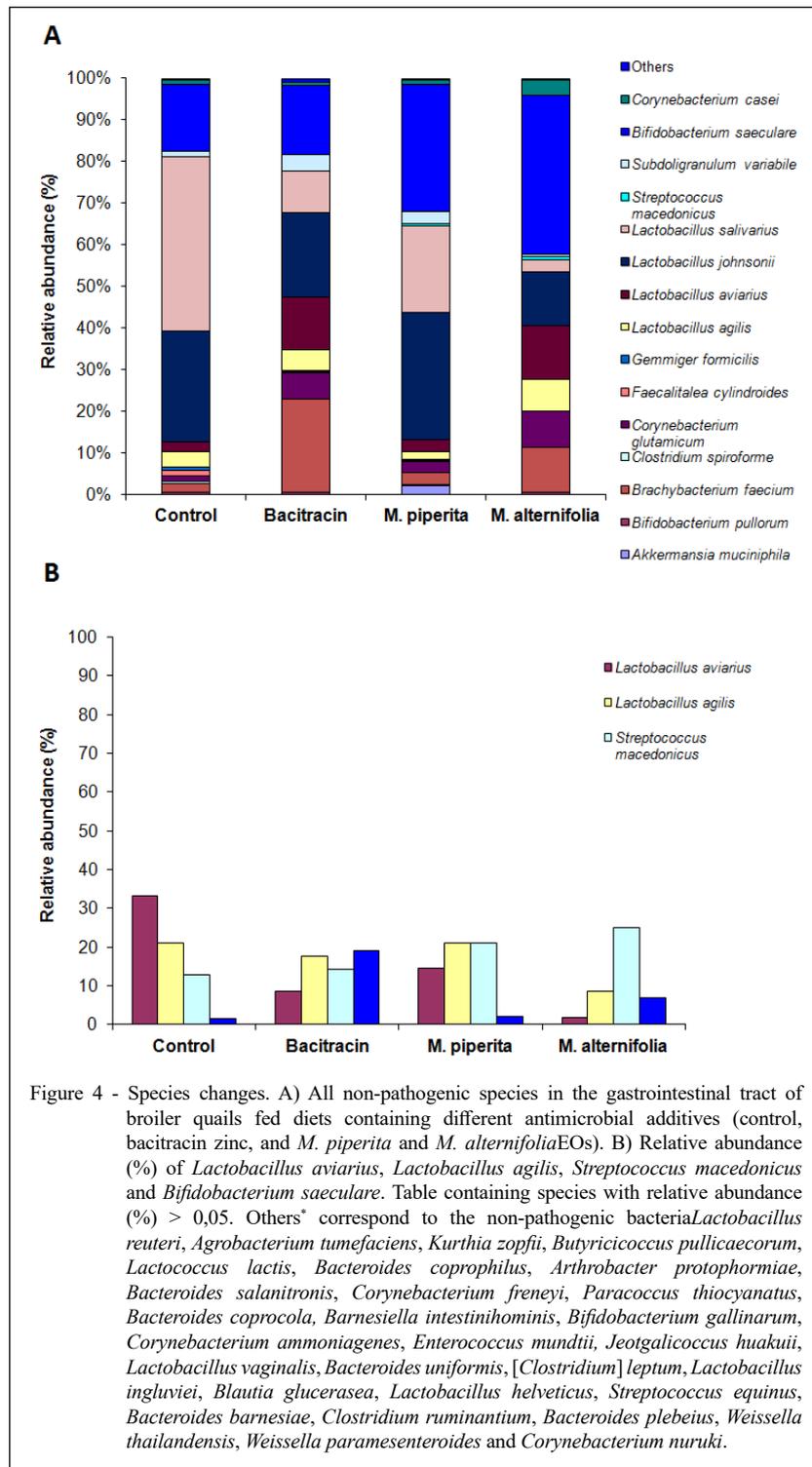
There was a significant decrease in *Lactobacillus aviarius* abundance for bacitracin (8.51%), *M. piperita* (14.44%), and *M. alternifolia* (1.80%) when compared with the control (33.31%).

Additionally, *Lactobacillus agilis* declined with the use of *M. alternifolia* (8.58%), but was found in higher percentages (21.07, 17.55, and 21.16%) in the remaining treatments (control, bacitracin, and *M. piperita*, respectively). There was also a significant increase in *Streptococcus macedonicus* abundance with *M. piperita* and *M. alternifolia* application (21.09 and 24.91%, respectively), and higher *Bifidobacterium saeculare* relative abundance in treatments with bacitracin (19.21%) and *M. alternifolia* (6.93%), whereas controls and *M. piperita* obtained values below 2% (Figure 4B).



CHANG et al. (2020) evaluated three types of probiotics (*Bacillus subtilis*, *Lactobacillus casei*, and *Candida utilis*) in broiler diets and found that the predominant intestinal bacterium was *Lactobacillus aviarius*, suggesting this species may play an important role in

reestablishing a new healthy microbial community, thereby mitigating mycotoxin toxicity in broilers. WIERSEMA et al. (2021) studied the intestinal permeability, morphology, and gut microbiome of laying hens and observed greater *Lactobacillus aviarius* and *Lactobacillus kitasatonis* abundance.



Quails treated with *M. alternifolia* exhibit greater intestinal bacterial diversity, separating this treatment from the others in cluster analysis (Figure 5A). Nine species belonging to four phyla were detected

exclusively in this treatment (Figure 5B), namely Phylum Firmicutes: *Staphylococcus haemolyticus*; Phylum Actinobacteria: *Bifidobacterium gallinarum*; *Corynebacterium frenyi*; Phylum Proteobacteria:

*Agrobacterium tumefaciens*; *Bosea thiooxidans*; *Luteimonas aestuarii*; *Paracoccus thiocyanatus*; *Serratia proteamaculans*; and Phylum Bacteroidetes: *Bacteroides salanitronis*. By contrast, quails treated with bacitracin zinc showed the lowest bacterial diversity, with only two species identified exclusively in this treatment (Figures 5A and B).

The gut microbiome has many functions and plays a significant role in quail health and performance WILKINSON et al. (2016). Changes in intestinal bacteria populations occur due to the adverse effect of antimicrobial agents that can alter the structure of the intestinal cell wall and influence the growth of pathogenic bacteria, a serious concern when using these agents in animal nutrition KÜREKCI et al. (2021). As such, it is desirable to maintain the balance of natural microbiome in the digestive tract.

#### Intestinal histomorphometry of broiler quails

The histological conformation of the GI tract is responsible for absorbing nutrients from ingested food, an important factor in host health because it directly impacts host immunity. The gut microbiome profile can influence intestinal morphology and morphometry and interfere in its function, thus altering nutrient uptake, among other issues. The gut morphology of the quails treated in the present study (control, bacitracin, *M. piperita*, and *M. alternifolia*) is shown in photomicrographs of the duodenum, jejunum, and ileum (Figure 5).

Changes in the microbiome profile of the intestinal lumen changes can benefit or harm the morphology and morphometry of the villi, causing alterations that directly affect their nutrient uptake and cell renewal rate. Figure 6 shows the specific characteristics of the different sections of the intestine (duodenum, jejunum, and ileum) according to the number and size of villi, crypt depth and muscle height.

Nutrient uptake occurs more in some sections of the intestine (the duodenum, for example) than others, resulting in different villus development levels and size in each section. In the present study, differences were also observed between equivalent sections depending on the treatment applied (control, bacitracin, *M. piperita*, and *M. alternifolia*), thereby altering the morphology of the intestinal epithelium.

Table 2 presents the results of the treatments (control, bacitracin, *M. piperita*, and *M. alternifolia*) in terms of gut morphometry. The inclusion of EOs and bacitracin zinc in the diet of broiler quails from 1 to 42 days old affected ( $P < 0.05$ ) intestinal sections and treatment-intestinal section interaction.

According to the data in the table 2, bacitracin and *M. alternifolia* obtained similar results for villus surface area, indicating an equivalent response to these two treatments for the duodenum. A larger villus surface area is positive for nutrient uptake. The coefficient of variation was 43.57%, revealing high data heterogeneity related to the treatment mean in the duodenum. This reveals the different effects of each antimicrobial, natural or otherwise, thereby increasing the variability of the gut morphological response. There were no statistical differences between the jejunum and ileum for the treatments tested, resulting in more homogeneous data (31%) than those obtained in the duodenum.

According to REZAEI et al. (2018), quails with a large villus surface area exhibit better nutrient uptake due to lower crypt cell proliferation. The results of the present study corroborated these data, since bacitracin and *M. alternifolia* EO increased the surface area of intestinal villi in the duodenum, albeit insufficient to reduce the number of crypt cells per villus.

With respect to villus height, the results obtained for bacitracin and *M. alternifolia* were

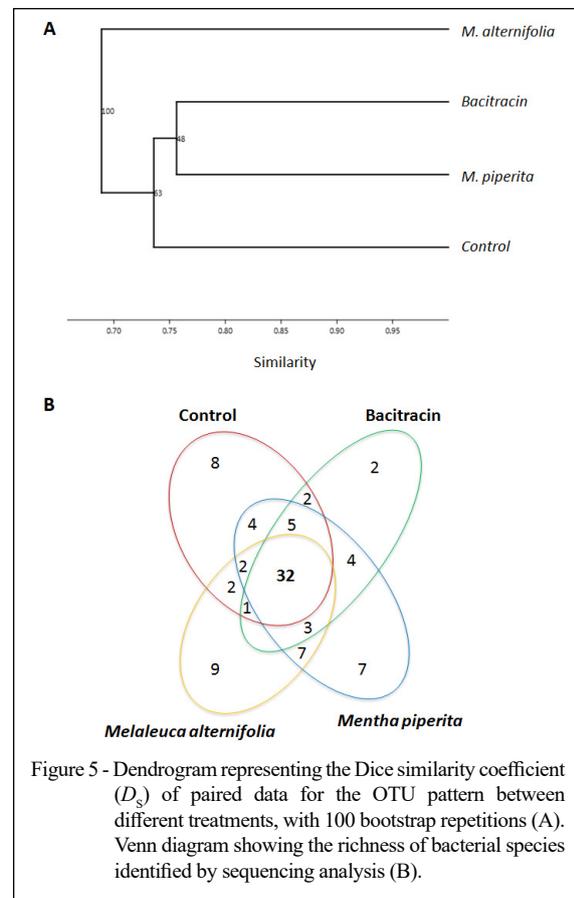
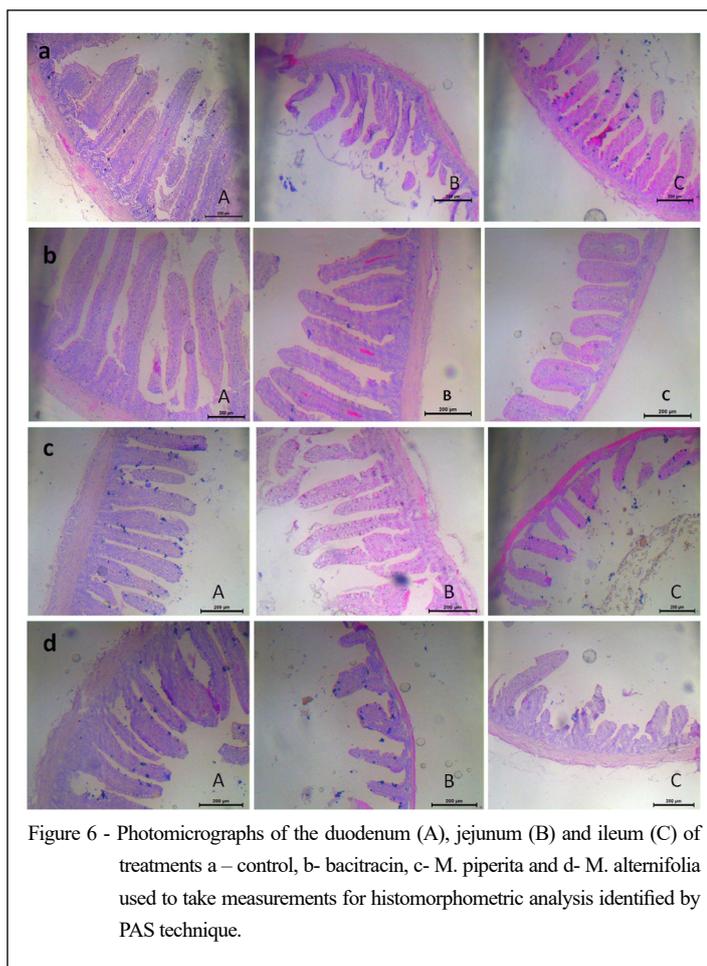


Figure 5 - Dendrogram representing the Dice similarity coefficient ( $D_s$ ) of paired data for the OTU pattern between different treatments, with 100 bootstrap repetitions (A). Venn diagram showing the richness of bacterial species identified by sequencing analysis (B).



statistically equivalent in the duodenum, with greater crypt depths also observed. In the jejunum, crypt depth in the two EO treatments was statistically superior. The coefficients of variation indicate homogeneous data between treatments, with a variation below 23%.

PELICANO et al. (2005) studied the effect of supplementation with two probiotics on gut morphology and observed that, in relation to control, both pro and prebiotics resulted in greater villus height in all three sections and increased crypt length in the duodenum and jejunum. BUENO et al. (2012) evaluated the influence of probiotic supplementation (*Bacillus subtilis*, *Aspergillus oryzae*, and *Saccharomyces cerevisiae*) on the gut morphology of quails and reported that probiotics reduced crypt depth but had no effect on villus height or the villus/crypt ratio.

According to DUNSFORD et al. (1989), the use of natural or chemical antimicrobials can cause changes in the small intestine, particularly reduced villus height and greater crypt depth and

cell proliferation. A considerable number of studies in the literature address the use of natural plant-based compounds to improve animal health and performance CASTILLO-LÓPEZ et al. (2017); SHAH et al. (2014). In this regard, an important practical concern in the successful use of plant-based compounds are side effects due to the considerable variation in their chemical components, including phenols, ethers, alcohols, esters, aldehydes and ketones KÜREKCI et al. (2021). However, orally administered EOs were not toxic to animal health at the doses used in our study. Additionally, results on the effectiveness of phytochemical compounds remain conflicting and inconsistent.

*M. alternifolia* and bacitracin zinc produced similar increases in villus surface area and height in the duodenum, resulting in a larger surface for nutrient intake in the small intestine. CARDOSO et al. (2012) studied the use of black pepper oil (*Piper nigrum*) in the diet of broilers and observed increases in villi of the duodenum and ileum (height vs width), also producing

Table 2 - Villus surface area, villus height, crypt depth, crypt to villus ratio, villus width x height ratio and intestinal muscle height in 42-day-old broiler quails fed diets containing EOs.

VILLUS surface area ( $\mu\text{m}^2$ )	-----Treatments-----				CV%	P-value
	Control	Bacitracin	M. piperita	M. alternifolia		
Duodenum	1381.87b	3427.06a	1342.58b	2385.28ab	43.57	0.0078
Jejunum	1584.20a	1422.45a	1745.58a	1854.94a	30.91	0.5738
ileum	1114.38a	1030.75a	792.99a	1002.97a	31.09	0.4203
Villus height ( $\mu\text{m}$ )	Control	Bacitracin	M. piperita	M. alternifolia	CV%	P-value
Duodenum	569.94b	981.82a	545.94b	841.23a	21.57	0.010
Jejunum	651.12a	620.80a	755.23a	682.36a	22.56	0.5591
ileum	507.07a	464.94a	398.27a	437.20a	15.7	0.1421
Crypt depth ( $\mu\text{m}$ )	Control	Bacitracin	M. piperita	M. alternifolia	CV%	P-value
Duodenum	80.48bc	99.64ab	73.43c	106.17a	21.78	0.0495
Jejunum	65.98b	67.42b	76.36ab	93.15a	16.66	0.0133
ileum	57.05b	65.56ab	75.90a	56.59b	11.96	0.0031
Crypt to villus ratio	Control	Bacitracin	M. piperita	M. alternifolia	CV%	P-value
Duodenum	2.42b	3.49a	2.46b	2.84ab	12.33	0.0470
Jejunum	2.43a	2.29a	2.31a	2.72a	16.36	0.1556
ileum	2.20a	2.22a	1.99a	2.29a	13.9	0.4799
Villus width x height ratio	Control	Bacitracin	M. piperita	M. alternifolia	CV%	P-value
Duodenum	2.30a	2.80a	2.28a	2.70a	12.75	0.0519
Jejunum	1.69a	2.04a	1.83a	2.13a	22.07	0.3776
ileum	1.60ab	1.50b	1.91ab	2.10a	12.69	0.0024
Intestinal muscle height	Control	Bacitracin	M. piperita	M. alternifolia	CV%	P-value
Duodenum	74.22b	105.60a	90.40ab	91.84ab	18.97	0.0075
Jejunum	76.14a	82.38a	82.07a	94.46a	25.78	0.6038
ileum	71.68a	83.06a	90.93a	73.02a	16.29	0.1022

\* $P < 0.05$ ; Means followed by the same lowercase letter do not differ according to Tukey's test at 5% probability.

a larger intestinal intake surface. VALLADÃO et al. (2017) assessed the intestinal morphology of fish (*Oreochromis niloticus*) fed with *M. alternifolia* EO and reported greater villus height compared to the other groups studied, with the larger intestinal surface leading to better nutrient intake and use.

With respect to crypt depth, the results for the duodenum were similar to those recorded for *M. alternifolia* and bacitracin and superior to the control. EMAMI et al. (2012) analyzed the effect of *M. piperita* (200 mg/kg) as an alternative to virginiamycin in broiler chickens and obtained analogous results for crypt depth in the duodenum for *M. piperita* and the antibiotic.

The results of the present study corroborated these findings; although, the *M. piperita* concentration used here was 12.5 mg/kg. This may be because higher crypt depth values indicate greater cell proliferation, thus ensuring an adequate epithelial

renewal rate LOPES et al. (2011). In this regard, the greater crypt depth may suggest rapid villus renewal, which increases the villus height to crypt depth ratio.

A high villus/crypt ratio may be related to adequate differentiation of the intestinal mucosa, improving absorption and reducing energy loss ARRUDA et al. (2008); JEURISSEN et al. (2002). Based on the results of the present study, the villus/crypt ratio was significant in the ileum. This corroborates the findings of REIS et al. (2016), who evaluated gut morphometry by including digestible threonine levels in the diet of growing broiler quails and recorded a higher villus/crypt ratio in the ileum at 42 days.

The height of the intestinal epithelium in broiler quails at 42 days old was not affected ( $P < 0.05$ ) by the treatments (EOs and bacitracin zinc) incorporated into their diet, the section of the intestine, and treatment-intestinal section interaction. The

musculature of the duodenum is strongly correlated with crypt depth and responsible for movement, assisting digestion and nutrient intake JUNQUEIRA & CARNEIRO (2008).

According to PAIXÃO & CASTRO (2016), the indiscriminate use of antibiotics can generate bacterial resistance and cause an imbalance in the gut microbiota. Alternatively, EOs act on the mucosa of the intestinal epithelium, contributing to the control and colonization of different species of pathogenic and non-pathogenic bacteria WILLIAMS et al. (2008). Thus, certain microorganisms in the GI tract may control microbiome composition BARBOSA et al. (2010) and promote positive changes in intestinal histomorphometry by improving nutrient uptake capacity.

The present study demonstrated the effect of EOs on the morphology of the small intestine of broiler quails. MEHRI et al. (2015) and KÜREKCI et al. (2021) reported that villus height and crypt depth increased significantly after *M. piperita* supplementation in the diet of quails, which did not occur for the same plant species in our study but was observed in the bacitracin and *M. alternifolia* treatments.

SAKI et al. (2017) reported a decline in villus height and greater crypt depth with the presence of toxic substances in the diet. These results indicated that none of the alternative antimicrobials had a harmful toxic effect to the point of reducing intestinal villus height.

## CONCLUSION

In summary, this study provided evidence that using *M. piperita* and *M. alternifolia* EOs in the diet of broiler quails modifies the intestinal microbiome, increasing bacterial diversity and reducing pathogenic bacteria. However, *M. alternifolia* EO promoted similar results to those obtained with the antibiotic bacitracin zinc, increasing the surface area and height of intestinal villi in the duodenum.

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## DECLARATION OF CONFLICT OF INTEREST

The authors declared that there are no conflicts of interest. The funding agency had no role in the study design, data collection, analysis or interpretation, or in the writing of the manuscript and the decision to publish the results.

## AUTHORS' CONTRIBUTIONS

All the authors contributed equally to the conception and writing of this manuscript. All the authors critically reviewed the manuscript and approved the final version.

## BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

All the procedures in this study were approved by the Animal Ethics Committee of Universidade Estadual de Santa Cruz (UESC, Ilhéus, BA, Brazil), under protocol number 016/18.

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