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Orange essential oil in the diet of broilers: performance, organ biometrics, bone characteristics, and intestinal morphometry

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ABSTRACT - Growth performance, organ biometrics, bone characteristics, and intestinal morphometry were evaluated in broilers fed a diet containing orange (*Citrus sinensis* L.) essential oil. A completely randomized design was used, with five treatments with orange essential oil (0, 100, 200, 300, and 400 mg kg^{-1} diet) and six replications with 20 birds per experimental unit. In the pre-starter phase, feed intake and weight gain of all birds linearly increased, while feed conversion decreased with the addition of orange essential oil in the feed. At day 21, bone density (Seedor Index) and body weight were higher in the birds that received the maximum level of essential oil (400 mg kg^{-1}) compared with those not treated with essential oil. The observed effects resulted from the better functioning of the physiological mechanisms of digestion and absorption of nutrients, characterized by the increase in villus height. Glycemia and weights of gastrointestinal tract organs of broilers at 21 days of age were not influenced by the evaluated essential oil. The results show that the addition of phytogenic additives to the diet does not cause any physiological impairment in birds.

Keywords: *Citrus sinensis*, jejunum, limonene, phytogenic additive, Seedor index

1. Introduction

The animal production industry has undergone countless and significant changes in recent years. There is an increasing concern with issues of food security for consumption, food impacts for human health, and bacterial resistance to the antimicrobials used as feed additives. Restrictions in the use of antibiotics to improve performance exacerbated the need of using alternative additives, such as phytogenics or phytobiotics (Seidavi et al., 2020).

Phytogenic additives are substances derived from medicinal plants or spices, such as essential oils, which have positive effects on production and health of animals. Beneficial results of phytogenic additives are attributed to the presence of diverse classes of active substances conferring antimicrobial actions and stimulus to digestion and promoting production of endogenous enzymes in animals (Erhan and Bölükbaşi Aktaş, 2017; Aydin et al., 2018; Seidavi et al., 2020; Sevim et al., 2020). There is also evidence that essential oils can impact pathogen concentrations in the intestine and improve feed digestibility (Micciche et al., 2019).

In recent years, interest in using essential oils in feed has been increasing. Citrus oils contain high quantities of limonene (El Sawi et al., 2019; Sahu et al., 2019; Erhan, 2020), to which most of their biological activities have been attributed, including antimicrobial (Bozkurt et al., 2017; Ambrosio et al., 2019), antioxidant, anti-inflammatory, analgesic or anti-nociceptive, antidiabetic, induction of osteogenesis, and effects on the gastrointestinal tract (Soulimani et al., 2019).

Essential oils affect bacterial proliferation in four ways: affecting the cell wall by removing phospholipids and obstructing ions of passive passage through the passage obstruction of active ions and inhibition of ATP synthesis, destruction of bacterial cytoplasm, and inhibition of energy synthesis in mitochondria (Chouhan et al., 2017; Namdeo et al., 2020).

Competitiveness in poultry farming is based on the obtention of better animal-performance indexes associated with the welfare and health of animals, as well as cost reduction and environmental impacts. Hence, it is relevant to study and evaluate the effect of different nutritional plans and breeding conditions on the physiology, biochemistry, and production of birds and combine these treatments to maximize benefits.

This study aimed to evaluate productive performance, organ biometrics, bone characteristics, and morphometry of the intestine of broilers fed a diet containing orange essential oil (*Citrus sinensis* L.).

2. Material and Methods

The procedures used in this study were approved by the Institutional Committee on Animal Use (case number 001/2018). The experiment was conducted in Pinheiral, Rio de Janeiro, Brazil (22°30'46" S, 44°00'02" W).

The statistical design was completely randomized, with five treatments, six repetitions, and 20 birds per experimental unit. The experiment utilized 600 commercial broilers from one to seven days of age. The treatments were 0, 100, 200, 300, and 400 mg of orange essential oil kg^{-1} diet.

The oil from the Pêra orange bagasse was obtained by hydrodistillation by using Clevenger equipment. The extraction yield was 4% and was determined through the ratio of the obtained oil mass by the plant mass used in the extraction (with an average of 100 g).

A gas chromatograph coupled with mass spectrometer (Shimadzu QP-2010 Plus) was used for separation, detection, and quantification of substances in the orange essential oil (Table 1). Essential oil (1.0 µL of 10 µL mL^{-1}) was injected into the chromatograph in splitless mode. The components were separated in a fused-silica capillary column (5% diphenyl and 95% dimethylsiloxane), length, internal diameter, and film thickness of 30 m × 0.25 mm × 0.25 µm, respectively. Helium was used as carrier gas with the flux of 1 mL min^{-1}. Temperatures of the injector and detector were 220 and 250 °C, respectively. The oven temperature was programmed at 60 °C for 2 min, with an increase of 5 °C min^{-1} until 110 °C, followed by an increase of 3 °C min^{-1} until 150 °C, and, finally, 15 °C min^{-1} until 290 °C, maintained constant for 15 min.

Table 1 - Chemical composition of Pêra orange (*Citrus sinensis* L.) essential oil

EO	RT	Substance	IRL_C	IRL_L	Concentration (%)
1	9.119	α-pinene	928	932	0.53
2	10.623	sabinene	968	969	0.32
3	11.287	myrcene	985	988	1.86
4	12.130	δ-3-carene	1006	1008	0.24
5	13.471	limonene	1036	1024	95.64
6	16.202	linalool	1096	1095	0.55
7	20.608	α-terpineol	1189	1186	0.87
Monoterpene hydrocarbon					98.59
Monoterpene oxygenated					1.41
Total of monoterpene					100.00

RT - retention time; IRL_C and IRL_L represent the linear retention index and literature index (tabulated), respectively.
Chemical composition was analyzed by GCMS (gas chromatograph coupled with mass spectrometer) and organized in the table by the elution order (EO) in the chromatographic column. The concentration (%) was calculated based on the total peak area by GCFID (gas chromatography with flame ionization detection).

Mass spectra were obtained using a quadrupole detector, operating at 70 eV with mass interval between 40-400 m z^{-1} and tax of 0.5 scan s^{-1}. Identification of substances in the essential oil was based on the comparison of retention indexes and mass spectra of samples with data from the library of National Institute of Standards and Technology (NIST 2008) and from literature (Adams, 2007). The Retention Index was calculated based on the co-injection of alkane samples of C8 to C40 as described in the literature (van Den Dool and Kratz, 1963).

During the initial experimental period, birds were heated using an automatic gas heater. Water was supplied by nipple drinkers and feed supplied by an infant tubular feeder, which was gradually replaced by a definitive tubular feeder. Wood shavings litter (10 cm deep) was used for floor covering of the aviary.

Lighting was maintained for 24 continuous hours (natural and artificial). The raising period was divided into two phases: pre-starter (1-7 days) and starter (8-21 days). Feeds were elaborated following the recommendations of Rostagno et al. (2017) (Table 2). Daily, we registered the mortalities that occurred and other management procedures necessary for the welfare of birds.

Table 2 - Composition of the experimental diets, in natural matter

Item	1-7 days	8-21 days
Ingredient (%)		
Soybean meal (44.15% CP)[1]	46.83	44.24
Maize (7.48% CP)[1]	44.80	47.18
Soybean oil	4.47	5.07
Dicalcium phosphate	1.90	1.07
Calcitic lime	0.75	1.22
Salt (NaCl)	0.53	0.51
DL-methionine	0.32	0.30
Vitamin premix[2]	0.10	0.10
Mineral premix[3]	0.10	0.10
Choline chloride	0.09	0.08
Inert material (washed sand)	0.05	0.05
L-lysine	0.04	0.06
L-threonine	0.02	0.02
Total	100.00	100.00
Calculated values[4]		
Metabolizable energy (kcal kg^{-1})	2,975	3,050
Crude protein (%)	24.27	23.31
Calcium (%)	0.92	0.88
Available phosphorus (%)	0.47	0.31
Chlorine (%)	0.38	0.37
Potassium (%)	1.00	0.96
Sodium (%)	0.22	0.22
Digestible lysine (%)	1.31	1.25
Total lysine (%)	1.45	1.39
Digestible methionine + cystine (%)	0.97	0.94
Total methionine + cystine (%)	1.06	1.02
Digestible methionine (%)	0.65	0.62
Total methionine (%)	0.67	0.65
Digestible threonine (%)	0.87	0.83
Total threonine (%)	0.99	0.95
Digestible tryptophan (%)	0.29	0.28
Total tryptophan (%)	0.33	0.31

CP - crude protein.
[1] Ingredient analysis.
[2] Guaranteed levels per kilo of product: vitamin A, 10,000,000 IU; vitamin D3, 2,000,000 IU; vitamin E, 30,000 IU; vitamin B1, 2.0 g; vitamin B6, 4.0 g; pantothenic acid, 12.0 g; biotin, 0.10 g; vitamin K3, 3.0 g; folic acid, 1.0 g; nicotinic acid, 50.0 g; vitamin B12, 15,000 mcg; and vehicle q.s.p., 1,000 g.
[3] Guaranteed levels per kilo of product: manganese, 16.0 g; iron, 100.0 g; zinc, 100.0 g; copper, 20.0 g; cobalt, 2.0 g; iodine, 2.0 g; selenium, 0.25 g; and vehicle q.s.p., 1,000 g.
[4] According to Rostagno et al. (2017).

Performance was evaluated in the phases described and the following characteristics were verified: feed intake (FI), weight gain (WG), feed conversion (FC), and viability. At the end of each period, birds from each experimental unit were weighed in groups, and the result was divided by the number of live birds, yielding the average WG and FI. Feed conversion ratio (FCR) was calculated by dividing FI by WG of birds. Viability was calculated using the total number of dead birds subtracted by total live birds.

At 21 days of age, a single fasting bird from each repetition (30 units in total) was taken for total blood glucose quantification (Rezende et al., 2019), with the use of a portable digital glucometer (G-Tech Lite®, South Korea). Then, the birds were slaughtered and their organs (liver, pancreas, gizzard, and intestines) removed and weighed immediately. The length of small intestine was measured with a tape.

Morphometric analyses of the intestine followed the procedures described in Faveri et al. (2015) and Erhan and Bölükbaşi (2017). Fragments of the birds' jejunum were collected and fixed in a 10% buffered formaldehyde solution. Then, segments were transferred to 70% alcohol, dehydrated in a growing series of alcohols, diaphanized in xylol, and embedded in paraffin. The slides with the cuts were stained using the hematoxylin and eosin technique. In the captured images, the villus height (VH) and crypt depth (CD) were measured using ImageJ. Villus height was measured from the basal region coinciding with the superior portion of the crypt to the tip of the villus, while the CD was measured from the basal region of the villus to the bottom of the crypt.

As regards bone analysis, in the tibiotarsus *in natura*, all adherent tissue was removed with the aid of scissors and tweezers, weighed on an analytical balance (±0.0001 g), and the diameters (horizontal and vertical) and length were measured using a digital pachymeter (0-150 mm, to an accuracy of 0.001 mm). The Seedor Index (SI; Seedor et al., 1991) was calculated by the bone weight (mg) divided by its length (mm).

In the analysis of bone resistance, bones were subjected to a flexion test, with the use of a texturometer (Stable Micro System, TA.XT.plus® model). All bones were tested in the same position, with the extremities resting on supports, and the load was applied in the central area (bone diaphysis). The value corresponding to bone rupture was expressed as kilogram-force (kgf).

Data were subjected to variance and regression analysis in SAS (Statistical Analysis System, version 9.0) software, using PROC REG with $\alpha = 0.05$. Additionally, a Dunnett's test ($\alpha = 0.05$) was used to compare the treatment without orange essential oil (control) to the other treatments.

The following statistical model was adopted:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij},$$

in which Y_{ij} = value observed for the variable response obtained for the *i*-th treatment (*i* = 0, 100, 200, 300, and 400 mg kg^{-1} of orange essential oil) in its *j*-th repetition, μ = general average, α_i = effect of treatment *i* on the observed value Y_{ij}, and ε_{ij} = random error related to each observation.

3. Results

The use of Pêra orange essential oil in rations influenced the pre-starter performance of broilers (Table 3). Broiler chicken FI, WG, and, consequently, the total weight at seven days of age increased in a linear trend. Feed conversion ratio was reduced with the addition of orange oil in rations, which indicates the beneficial effect of the additive.

At 21 days of age, broilers that received 400 mg kg^{-1} of orange essential oil showed higher body weight (BW) compared with birds fed diets without essential oil (Table 4, $P<0.05$).

The addition of orange essential oil in diets had no influence on the weights of gastrointestinal tract organs and glucose of broilers at 21 days of age (Table 5). Bone characteristics of broilers (Table 6) were modified with the addition of Pêra orange essential oil in diets. The *in natura* weight and length

were higher in the tibia of birds that received, respectively, 300 and 400 mg kg^{-1} of orange essential oil, when compared with the treatment without the essential oil (P<0.05).

The morphometry of the jejunum of broilers fed diets with orange essential oil was significantly changed (Table 7). Villus height was adjusted in increasing quadratic model (P<0.05), which indicated an increase in the absorption surface in the intestine, demonstrated by the higher CD in birds at 21 days of age.

4. Discussion

The use of Pêra orange (*Citrus sinensis* L.) essential oil in diets influenced the pre-starter performance of birds (Table 3). A lower FCR value was observed, similar to findings of Erhan and Bölükbaşi (2017), who studied the inclusion of citrus oils (bergamot, orange, and lemon) in diets (1, 2, and 3 mL kg^{-1}) for broilers. The authors obtained positive results in FCR with the use of orange essential oil. Similarly, Aydin and Alçiçek (2018) evaluated orange essential oil (0, 50, 100, and 150 mg kg^{-1}) in rations of broilers until six weeks of age. These authors recommended the quantity of 150 mg kg^{-1}, since higher rates of productive efficiency were verified.

Table 3 - Performance of broilers from one to seven days of age fed diets containing orange (*Citrus sinensis* L.) essential oil

Variable	Orange essential oil (mg kg^{-1})					CV (%)	P-value
	0	100	200	300	400		
Initial weight (g)	40.37	40.12	39.95	39.95	40.16	1.20	-
Weight at seven days (g)[1]	117.96	138.67	143.37	152.08	159.87	4.62	<0.05
Total WG (g $bird^{-1}$)[2]	77.58	98.54	103.42	112.12	119.71	6.40	<0.05
Daily WG (g $bird^{-1}$ day^{-1})[3]	11.22	14.08	14.77	16.01	17.10	6.45	<0.05
Total feed intake (g)[4]	81.21	97.12	99.83	106.41	109.92	6.62	<0.05
Feed intake (g $bird^{-1}$ day^{-1})[5]	11.60	13.88	14.26	15.20	15.70	6.62	<0.05
Feed conversion ratio[6]	1.04	0.98	0.97	0.95	0.92	6.65	<0.05

CV - coefficient of variation; WG - weight gain.
[1] $\hat{Y} = 122.94167 + 0.09725x$; $R^2 = 0.8239$.
[2] $\hat{Y} = 82.70833 + 0.09783x$; $R^2 = 0.8269$.
[3] $\hat{Y} = 11.90133 + 0.01369x$; $R^2 = 0.8179$.
[4] $\hat{Y} = 85.55833 + 0.06671x$; $R^2 = 0.6898$.
[5] $\hat{Y} = 12.22267 + 0.00953x$; $R^2 = 0.6893$.
[6] $\hat{Y} = 1.02733 - 0.00027x$; $R^2 = 0.2712$.

Table 4 - Performance of broilers from one to 21 days of age fed diets containing orange (*Citrus sinensis* L.) essential oil

Variable	Orange essential oil (mg kg^{-1})					CV (%)	P-value
	0	100	200	300	400		
Initial weight (g)	40.37	40.12	39.95	39.95	40.16	1.20	-
Weight at seven days (g)[1]	117.96	138.67	143.37	152.08	159.87	4.62	<0.05
Weight at 14 days (g)	327.32	357.72(+)	354.73(+)	380.17(+)	399.73(+)	4.66	<0.05
Weight at 21 days (g)	705.39	711.63	668.54(-)	711.66	766.46(+)	5.23	<0.05
Total WG (g $bird^{-1}$ day^{-1})	665.01	671.51	628.58(-)	671.70	726.29(+)	5.53	<0.05
Weight gain (g $bird^{-1}$ day^{-1})	31.67	31.97	29.93(-)	31.99	34.59(+)	5.52	<0.05
Total feed intake (g $bird^{-1}$)	860.43	985.64(+)	899.16	995.70(+)	1082.45(+)	9.79	<0.05
Feed intake (g $bird^{-1}$ day^{-1})	40.97	46.93(+)	42.82	47.41(+)	51.54(+)	9.79	<0.05
Feed conversion ratio	1.29	1.47(+)	1.43	1.48(+)	1.49(+)	8.85	<0.05
Viability (%)	95.83	97.50	97.50	98.33	99.17	2.75	>0.05

CV - coefficient of variation; WG - weight gain.
[1] $\hat{Y} = 122.94167 + 0.09725x$; $R^2 = 0.8239$.
Averages followed by (+) were higher than the absolute control at 5% of probability by the Dunnett's test.
Averages followed by (-) were lower than the absolute control at 5% of probability by the Dunnett's test.

Table 5 - Organ biometrics and glucose of broilers at 21 days of age fed diets containing orange (*Citrus sinensis* L.) essential oil

Variable	Orange essential oil (mg kg^{-1})					CV (%)	P-value
	0	100	200	300	400		
Glucose (mg dL^{-1})	225.33	262.50	255.00	254.17	264.17	20.20	0.30
Bird weight (g)	690.00	704.17	670.00	721.67	766.17(+)	5.26	>0.05
Gizzard (g)	26.42	27.55	26.07	27.28	26.53	12.92	0.99
Pancreas (g)	2.31	2.32	2.39	2.43	2.40	12.65	0.44
Liver (g)	16.88	16.10	15.93	15.53	17.47	10.67	0.79
Small intestine (g)	32.54	30.82	29.84	32.79	32.47	11.46	0.70
Large intestine (g)	1.28	1.70	1.43	1.51	1.51	52.76	0.79
Small intestine length (cm)	143.67	140.92	142.33	138.37	142.99	9.10	0.81
Large intestine + cecum (g)	5.15	6.85	5.97	5.46	6.12	41.67	0.86
Cecum (g)	3.87	5.15	4.54	3.95	4.61	42.48	0.90
Gizzard (%)	3.83	3.92	3.90	3.77	3.45	12.08	0.13
Pancreas (%)	0.33	0.33	0.36	0.34	0.31	13.19	0.56
Liver (%)	2.45	2.29	2.38	2.15	2.28	10.33	0.12
Small intestine (%)	4.71	4.40	4.45	4.53	4.23	10.05	0.16
Large intestine (%)	0.18	0.24	0.21	0.21	0.20	53.96	0.94
Cecum (%)	0.55	0.73	0.68	0.55	0.61	43.39	0.83

CV - coefficient of variation.
Averages followed by (+) were higher than the absolute control at 5% of probability by the Dunnett's test.

Table 6 - Characteristics of tibias of broilers at 21 days of age fed diets containing orange (*Citrus sinensis* L.) essential oil

Variable	Orange essential oil (mg kg^{-1})					CV (%)	P-value
	0	100	200	300	400		
In natura weight (g)	6.22	6.66	6.26	7.00(+)	7.24(+)	9.28	>0.05
Length (mm)	70.54	72.53	72.48	73.71(+)	74.36(+)	2.60	>0.05
Horizontal diameter (mm)	5.72	5.42	5.24	5.50	5.89	7.39	0.42
Vertical diameter (mm)	5.37	4.92	4.65	4.82	5.26	11.60	0.66
Breaking strength (kgf cm^{-2})	15.06	13.12	10.72	10.65	14.03	32.92	0.40
Seedor Index	88.10	91.67	86.32	94.93	97.22(+)	7.42	>0.05
Dry weight (g)	2.16	2.31	2.17	2.43	2.51(+)	8.87	>0.05
Ash in tibia (g)	0.80	0.79	0.72	0.82	0.85	8.90	0.13
Ash in tibia (%)	36.42	35.10	34.23	35.12	34.93	8.02	0.42

CV - coefficient of variation.
Averages followed by (+) were higher than the absolute control at 5% of probability by the Dunnett's test.

Table 7 - Morphometry of the jejunum of broilers at 21 days of age fed diets containing orange (*Citrus sinensis* L.) essential oil

Variable	Orange essential oil (mg kg^{-1})					CV (%)	P-value
	0	100	200	300	400		
Villus height (VH; µm)	624.30	625.68	646.84	658.77	767.21(+)	10.71	>0.05
Crypt depth (CD; µm)	58.77	60.00	64.95	68.56	72.00(+)	10.95	>0.05
VH:CD	10.66	10.45	10.07	9.62	10.80	11.87	0.47

CV - coefficient of variation.
Averages followed by (+) were higher than the absolute control at 5% of probability by the Dunnett's test.

The pre-starter phase constitutes a critical stage in poultry farming, since the animals have more accelerated physiological, metabolic, and body development. It is equivalent to ¼ of the production cycle of broilers, being decisive in the profitability of the enterprise. The performance results in the referred phase denote the differentiation of enterocytes, as well as the capacity of absorption and transport of nutrients and production and secretion of digestive enzymes.

Further, a Dunnett's test was run to identify possible differences between the control treatment (without orange oil) and those containing different amounts of the phytogenic additive (orange essential oil). It was found that broilers at 21 days of age receiving 400 mg kg^{-1} of orange essential oil in their diet had higher BW ($P<0.05$). The highest WG and BW resulted from the improvement in the physiological processes in the digestive tract, as well as the possible inhibition of intestinal pathogenic organisms in the evaluated raising period (Table 4). Thus, the better feed efficiency achieved with the use of orange essential oil may be due to positive effects on the promotion of endogenous enzyme activity, as well as the antimicrobial activity of orange essential oil (Aydin and Alçiçek, 2018) and nutrient digestibility and intestinal health (Sahu et al., 2019).

The mechanism of action of essential oils on bacterial cells comprises a series of events that can destabilize the cellular architecture, leading to the breakdown of membrane integrity and, thus, increased permeability of the cellular constituents (Ambrosio et al., 2019). This disrupts many cellular activities, including energy production, membrane transport, and other metabolic regulatory functions. Furthermore, this action can alter the membrane fatty acid composition and membrane proton motive force and affect proteins in the cytoplasmatic membrane (Ambrosio et al., 2019).

The main constituent of the orange essential oil is limonene (95.64%), a terpene with the ability to release short-chain fatty acids (fermentation products), which lower the pH and prevent the growth of harmful microorganisms. Bozkurt et al. (2017) characterized different citrus essential oils obtained by the hydrodistillation method. The authors showed that the amount of limonene in each essential oil was directly proportional to the antimicrobial effect (*E. coli*, *B. cereus*, *S. aureus*, *S. Thyphimurium*, *E. faecalis*, and *L. monocytogenes*).

Further, orange essential oil contains myrcene, sabinene, α-sabinene, linalool, and other compounds in minor amounts (Table 1). The antimicrobial properties of orange essential oil can be assigned to each constituent feature of isolation (Guimarães et al., 2019) and also the synergy that occurs between them (Ambrosio et al., 2019; Berdejo et al., 2020). Ambrosio et al. (2019) demonstrated that limonene could collaborate with the selective activity of citrus oils when present in the gut, promoting the beneficial bacteria, while other minor compounds could act to inhibit pathogenic bacteria.

Regarding health challenge, birds were raised on reused litter. The viability in the pre-starter phase was 100%, and in the total period of the experiment (one to 21 days of age), the viability was not significantly affected with the dietary supplement of orange essential oil. In addition to the superior results on nutrient digestibility and the consequent performance of poultry (Aydin et al., 2018; Sahu et al., 2019), the use of phytogenics could improve viability. However, in this study, there was no change in this productive parameter, similar to findings reported by Aydin and Alçiçek (2018).

The orange essential oil evaluated did not influence the weights of the gastrointestinal tract organs and glucose of broilers at 21 days of age (Table 5). Similarly, Erhan and Bölükbaşi (2017) and Aydin and Alçiçek (2018) did not observe any changes in the liver weight of broilers fed citrus oils at 42 days of age. Dhanapal et al. (2014) studied essential oil of citrus fruits (2.5 g kg^{-1}) in diets, with or without aflatoxin for broilers, and did not find adverse effects of the essential oil on relative weight of lymphoid organs, kidneys, heart, and serum biochemistry of healthy birds.

Biometrics of digestive system organs is an instrument to be used in physiological and economic evaluation of the use of additives in diets. The viscera are commercially important and can be used as a signal for metabolic effects of dietary manipulation. Weights and percentages of the intestines (small and large), as well as length of the small intestine, were not changed by the treatments, which demonstrate no physiological changes of birds.

Glycemic levels were within the normal range (200-500 mg dL^{-1}) for healthy birds (Schmidt et al., 2007) or birds that received the orange essential oil (Sevim et al., 2020). In an experiment by Karabayir et al. (2018), broilers fed diets containing 600 ppm of orange essential oil (*Citrus sinensis*, 94.74% of limonene) exhibited high serum glucose values. The authors reasoned that there was a breakdown of glucose mobilization of the tissues and, thus, the molecules migrated from the tissues directly into the blood. The maximum quantity of orange essential oil evaluated (400 mg kg^{-1}) was safe and possibly insufficient to cause a change in the glycemia of the animals.

The *in natura* weight and length ($P<0.05$) were higher in the tibia of chickens that received 300 and 400 mg kg^{-1} of orange essential oil in their diet, demonstrating the effects of phytogenics on mineral metabolism. Sevim et al. (2020) observed a serum modification of calcium and phosphorus (increase) in broilers given orange essential oil, likely because of limonene, which lowers the pH of the digestive system, increasing endogenous digestive enzymes and the intestinal surface area, thereby, enabling improvement in mineral absorption that was observed in this assay.

Seedor Index indirectly indicates bone density, and a higher index shows a higher density of the bone and vice versa. Broilers fed 400 mg kg^{-1} of orange essential oil had a higher dry weight and density in the tibia (SI), when compared with birds that did not receive the essential oil (Table 6). Sabbieti et al. (2011) described that essential oils have lipophilic properties, cross cell membranes easily, and affect the function of bone cells by stimulating or inhibiting specific metabolic pathways. Furthermore, they can modulate the proliferation of osteoblasts, probably by alternative signaling, which depends on the maturation stage of the cells, and increases mineral density and bone strength (Olgun, 2016). Limonene can be considered a promising compound for bone healing through the induction of osteogenesis (Soulimani et al., 2019).

The intestinal microbiota of birds has probably influenced bone variables (*in natura* weight, length, and SI) in the present study. According to Lunedo and Pedroso (2017), the effects of the microbiota on bone mass occur via the immune system, particularly from the role of T cells, which regulate the formation of osteoclasts. In addition, the presence of microorganisms can impact mineralization, density, and bone strength. No changes were observed in mineral composition (ash) and in the breaking strength of the tibia; however, the density (SI) was higher with the use of orange essential oil, which showed the integrity in the filling of the bone matrix.

The morphometric characteristics of the broiler jejunum were significantly altered (Table 7). It was found that the villus height showed a quadratic response, indicating an increase in the absorption surface in the intestine, also confirmed by the greater depth of crypts. Erhan and Bölükbaşi (2017) also found differences in the histological variables of the jejunum (increase in villus length) of chickens, when using orange essential oil in diets (3 mL kg^{-1}).

The increase in height of the villi observed can also be correlated with the antioxidant properties of the essential oil studied. According to Namdeo et al. (2020) oxygen radicals released during digestion attack the superficial mucosa of the intestine and can shorten the intestinal villi. Antioxidant enzymes such as catalase, glutathione peroxidase, and superoxide dismutase can attract oxygen radicals and extinguish free radicals (Namdeo et al., 2020). Limonene, a constituent of orange essential oil, prevents oxidative damage and can protect lymphocytes against oxidative stress and stimulate cell proliferation (Soulimani et al., 2019).

The long villi correlate with the improvement of the intestinal health of birds, which provides better uniformity and integrity of the mucosa, besides the greater the capacity to absorb nutrients (Borsatti et al., 2020). The intestinal mucosa must have adequate morphofunctional characteristics since the absorption processes depend on the integrity of the epithelium. Thus, stimulation in the development of the intestinal mucosa, with trophic agents in the diet such as essential oils, has become an important nutritional strategy.

The use of orange essential oil in rations also influenced the CD, the value of which was greater in treatments of 300 and 400 ppm. The verified results may be due to the possible modulation of the

immune system resulting from limonene (Wang et al., 2019), as well as the greater need for renewal of the intestinal epithelium. Bayrakdar et al. (2017) investigated histomorphometric characteristics and endocrine cells immunoreactive to serotonin (CEIR) in crypts of the jejunum of Japanese quails under heat stress and fed diet containing the orange essential oil (300 ppm). Their results showed an increase in CEIR and in CD of the jejunum with the orange essential oil supplement.

The increase in CD can denote an accelerated villus renewal rate, which leads to a greater energy expenditure by the cells. According to Faveri et al. (2015), the mitotic divisions in the crypts account for about 60% of cell proliferation, the middle region of villus is responsible by 32% of cell proliferation, and the apical region by 8%.

The VH:CD ratio was not influenced by the essential oil levels ($P<0.05$) added to the diets. This ratio is a good indicator of the proliferation and development of enterocytes in the villi. In other words, the digestive efficiency of the animal and a decrease in this ratio is not acceptable in terms of digestion and absorption, and vice versa (Namdeo et al., 2020). The desirable relationship occurs with high villi and shallow crypts, since there would be less energy losses with cell renewal and better nutrient absorption.

5. Conclusions

The addition of Pêra orange (*Citrus sinensis* L.) essential oil in diets promotes the improvement of productive variables (weight gain, feed intake, and feed conversion) of broilers in the pre-starter phase. At 21 days of age, birds that received 400 mg kg^{-1} of orange essential oil showed higher weight gain and, consequently, higher body weight. The verified results are a consequence of the improvement in physiological processes in the digestive tract, which is characterized by the increase in villus height. Bone characteristics are influenced by the phytogenic additive, with an increase *in natura* weight, length, and bone density. The biometrics of gastrointestinal tract organs and glycemia of birds are not altered by the treatments, indicating that there is no physiological impairment to the birds.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Data curation: C.S. Souza and A.S. Chaves. Formal analysis: F.M. Vieites and A.S. Chaves. Project administration: C.S. Souza, F.M. Vieites, C.S. Minafra and C.A.R. Lima. Resources: M.F. Lima and C.S. Minafra. Supervision: F.M. Vieites, C.S. Minafra and C.A.R. Lima. Validation: C.S. Souza and L.R. Justino. Visualization: C.S. Souza and L.R. Justino. Writing-original draft: C.S. Souza.

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