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Original Article

# Natural Growth Promoters Replacing Traditional Growth Promoters in Diets for Light Replacement Pullets: A Systematic Approach

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## ABSTRACT

The effect of using natural growth promoters (NGP) to replace traditional antimicrobials on performance, biometry of digestive and reproductive organs, sexual maturity and bone characteristics of replacement pullets was evaluated; and the relationship between these variables according to the diets was verified. Eight-week-old birds were randomly assigned to a completely randomized design and fed different diets: negative control (without growth promoters); positive control - conventional growth promoter; organic acids (OA); symbiotic (S); essential oil (EO); OA + S; and EO + S. The performance, relative weight of digestive and reproductive organs and length intestines, height and crest length, sternum length, bone quality and sexual maturity of birds were similar ($p$>0.05) between treatments. The heat map combined with cluster analysis showed a uniform static pattern with the formation of three horizontal groups formed by the treatments: 1) negative control, S and OA + S; 2) positive control and OE and 3) OA and OE + S. A null relationship between the treatments and the variables under study was observed. The principal components analysis revealed an association of variables in three components with 60.55% of variation. NGP can replace traditional promoters, as they do not interfere with performance, biometrics or sexual maturity. Height and length are predictive variables for the development of reproductive organs, especially the oviduct. A similarity was identified through multivariate techniques between symbiotic and organic + symbiotic acids; positive control and essential oils; and organic and symbiotic acids + essential oils.

## INTRODUCTION

An increase in the production and commercialization of eggs has occurred in the poultry segment in the last decades, motivating studies which provide relevant information about the nutrition of spare pullets (Sena *et al.,* 2020), mainly for developing long-lived and high-productivity commercial layers (Farias *et al.,* 2020). The body and reproductive development of pullets depends on the ingestion of an appropriate amount of nutrients for body tissue deposition and for forming the reproductive system (D´Agostini *et al.,* 2017), providing adequate body conditions to express all of their genetic potential (Braz *et al.,* 2011). The balance of the intestinal microbiota of replacement pullets is essential to enhance this development (Neijat *et al.,* 2019), providing a better absorption of nutrients from the diet (Rebollada-Merino *et al.,* 2019).

Growth-promoting antibiotics have been used in the past few years in poultry diets aiming to improve health and establish the intestinal ecosystem, having greatly contributed to poultry development. However, such additives came to be seen as causing risks to human health due to the concern with the presence of residues in meat and eggs, and with the induction of cross resistance by pathogenic bacteria in humans (Thelma *et al.,* 2019). As a result, several countries have banned the use of antibiotics as growth promoters in poultry production, replacing them with natural additives which cause similar effects to traditional promoters (Gadde *et al.,* 2017). Thus, symbiotics, phytogenics and organic acids stand out among the possible natural substitutes in the global poultry market.

These natural promoters have different mechanisms of action, i.e. organic acids promote acidification of the diet with a reduction in stomach pH, reducing the proliferation of undesirable microorganisms (Ribeiro *et al.,* 2008). Carvacrol is an essential oil that is classified as a phytogenic additive, and controls the potential of pathogens and it's beneficial modulation of the intestinal microbiota as its main action modes (Murugesan *et al.,* 2015). Symbiotics are the combination of prebiotics and probiotics, and provide synergistic action in improving the absorption of nutrients and decreasing energy costs for replacing intestinal cells (Reis & Vieites, 2019). Studies have revealed that the synergistic action of combining these promoters endorses greater egg/meat production (Tang *et al.,* 2017; Wang *et al.,* 2019; Stefanello *et al.,* 2020).

In addition, these growth promoters, symbiotics, organic acids and essential oils, alone or in combination, provide similar results to those obtained with conventional promoters, and can be used in the diet of birds to replace them in all breeding stages, including in rearing with replacement pullets, optimizing performance, bone characteristics, the development of digestive and reproductive organs (Ribeiro *et al.*, 2010; Bastos-Leite *et al.*, 2016; Vasconcelos *et al.*, 2016). Thus, the use of these additives will provide efficient digestion and absorption of nutrients, ensuring better maturation of the birds' reproductive organs, as well as the crests and development of the sternum, ensuring high productivity (Jardim Filho *et al.*, 2011; WANG *et al.*, 2019).

Considering the potential use of natural additives in poultry, as well as the need for suitable substitutes for conventional growth promoters and the scarcity of research relating these additives to birds in the early production stages, this study was carried out with the objective of evaluating the effect of using natural growth promoters compared to the conventional growth promoter in diets for replacement pullets on their performance, biometrics of digestive and reproductive organs, bone characteristics and sexual maturity, and to verify the relationship between performance, biometrics and sexual maturity according to treatments using multivariate analysis techniques as an auxiliary method.

## MATERIAL AND METHODS

### Study location

The experiment lasted five consecutive cycles of 28 days each, and was carried out at the Poultry Posture Sector of the Vale do Acaraú Experimental Farm - FAEX, Sobral, Ceará, Brazil (3° 36" S, 40° 18" W and 56 m at sea level). The region's climate is BSh type (B-Dry, S-Semi-arid, and h- Low latitude and altitude - Köppen Climatic Classification) (Alvares *et al.,* 2013). The average temperature is 27 °C, 68.67% humidity, and an annual precipitation of 808 mm year$^{-1}$ concentrated in the first five months of the year (INMET, 2021).

### Facilities and equipment

The birds were housed in galvanized wire cages with dimensions of 90 × 45 × 45 cm with three subdivisions of 30 cm and a population density of 450 cm² bird$^{-1}$, with a front feeder on a metal channel (Zatti, *Santa Catarina*, Brazil) and a Nipple drinker (Dutch, Brazil), per cage. The shed was made of masonry with wire mesh, concrete floor and ceramic tile cover with East-West orientation containing 12 m long × 8 m wide and 2.60 m high ceiling.

### Animals, experimental design and diets

In the preparation of the experimental animals, all pullets from housing to 1 day before the start of the experiment (0 days to 8 weeks of age) were raised on the floor on a bed of wood scraps, where they had daily contact with their own excreta. All handling in the rearing and rearing phase was carried out in accordance with the lineage manual (Lohmann LSL Lite, 2016). They were only housed in suspended cages in the shed where the experiment took place at the age of 9 weeks. This shed had previously been populated by another batch of birds which had been discarded days before receiving the new batch of pullets. Meat and bone meal was used as a source of phosphorus in the diets throughout the experiment.

In addition, the pullets went through all the programmed handlings throughout the experimental period, such as individual vaccinations (where the birds were handled one at a time) and surgical beak trimming (at 11 weeks of age), which can cause a drop in resistance on part of the animals.

A total of 378 pullets from the Lohmann LSL Lite strain, 8 weeks old, clinically healthy and body weight 653.72 ± 6.11g were used in the experiment. The birds were weighed and selected to obtain experimental plots with uniform average weight according to recommendations proposed by Sakomura & Rostagno (2007). A completely randomized design with seven treatments and six repetitions, totaling 42 experimental units with nine birds each was adopted in the study. The treatments consisted of a basal diet - negative control ($N_C$), without additives, and six diets with different growth promoters and their combinations: Positive Control (PC) - Conventional growth promoter; Symbiotic (S); Organic acids (OA); Essential oil (EO); S + OA; S + EO.

The probiotic in the symbiotic used was composed of lactic acid-producing bacteria strains $10^4$ CFU/ml and the prebiotic was composed of mannan-oligosaccharides 370g/kg. The mixture of organic acids and their concentrations were fumaric acid (min.) 160 g/kg, sorbic acid (min.) 50 g/kg, malic acid (min.) 50 g/Kg, and citric acid (min.) 70 g/kg. The active component of the essential oil used was carvacrol – min. 250 mg/kg. Commercial information, inclusion levels and guarantee and manufacture of the additives used in the treatments are presented in Table 1.

**Table 1** – General characteristics of the commercial additives used in the study.

| Commercial name | Warranty Levels |
|---|---|
| Probiotic | Total anaerobic bacteria (104.0 CFU/mL)<br>Lactic acid-producing bacteria (104.0 CFU/mL)<br>Mannanoligosaccharides (370 g/kg) |
| Organic acid | Smoke acid (160 g/kg)<br>Sorbic acid (50 g/kg)<br>Malic acid (50 g/kg)<br>Citric acid (70 g/kg) |
| Essential oil | Carvacrol (min.) 250,0000 mg/kg |
| Antibiotic | Halquinol (60.0 g) |

The birds received the same daily handling during the experimental period. The supply of drinking water was *ad libitum*. The experimental diets (Table 2) were formulated according to the recommendations of the Lohmann LSL Lite Lineage Manual (Lohmann LSL Lite, 2016) and the composition of the ingredients used in the formulation followed the recommendation of Rostagno *et al.* (2017). Additives were added to the basal formulation to replace the inert (washed sand) formulation to compose the treatments.

The birds received natural lighting from the 10th week until starting production, and the night light program started when the birds reached 5% of production, in which one hour of light per week was added for a total of 4 hours, totaling 16 hours (12 daytime and 04 nighttime).

## Data collection

### *Performance*

The performance variables evaluated were average weight (g), weight gain (g), feed intake (g/bird/day) and feed conversion (g/g). The birds and diets were weighed every 28 days during the first two experimental periods to calculate these variables.

### *Biometrics of digestive and reproductive organs*

Next, 35 birds were randomly selected at 17 weeks, totaling 5 birds per treatment. They were euthanized by the universal method of cervical dislocation in accordance with Normative Resolution Nº 37/2018 of the National Council for the Control of Animal Experimentation (CONCEA). The biometrics of digestive organs (proventricle, gizzard, liver, pancreas, intestines and length of the intestines) and reproductive organs (ovary and oviduct) were subsequently performed, which were properly emptied and weighed individually using a high-precision digital scale (± 0.01 g, Maximum capacity: 500 g; MH-267-5; Chinese) and measuring tape to measure the length of the intestines (cm). All weight data were expressed as a percentage of body weight. Height (cm), crest length (cm) and sternum bone length (cm) were measured with a digital caliper (± 0.01 cm, maximum measurement: 150 cm, Chinese).

### *Assessment of sexual maturity*

The average age of the birds (weeks) at the first egg was verified when the birds in each experimental plot reached 50% and 100% of egg production.

### *Bone characteristics*

Bone quality was assessed using the birds' left tibiae and measuring the length in millimeters (mm) with a digital caliper (± 0.01 cm, maximum measurement: 150 cm, Chinese) to calculate the Seedor index. Bone density was assessed using the Seedor index by dividing the bone length (mm) by the weight of bone ash expressed in mg/mm (Seedor *et al.,* 1991). A mechanical press (Ronald Top LTDA, *Rio de Janeiro*, Brazil) was subsequently used to analyze bone resistance and deformity, with the tibias being placed in a horizontal position with a compression force being applied in the center of each bone. The maximum amount of force applied to the bone until rupture was considered to be the resistance to breaking (kgf/cm²), as measured by a digital extensometer. Deformity (mm) was measured using an analog extensometer until each bone ruptured.

**Table 2 -** Nutritional composition of the negative control diet of laying pullets in the periods from 8 to 12 weeks, 13 to 17 weeks and 18 to 28 weeks of age of age.

| Ingredient (g. kg$^{-1}$) | Periods | | |
|---|---|---|---|
| | 8 to 12 weeks | 13 to 17 weeks | 18 to 28 weeks |
| Corn grain (8.58%) | 596.223 | 595.141 | 595.126 |
| Soybean meal (45%) | 232.944 | 181.805 | 228.931 |
| Wheat bran | 100.000 | 150.000 | - |
| Meat and bone meal (41%) | 54.02 | 54.844 | 59.798 |
| Limestone | 5.460 | 7.972 | 8.3435 |
| Soybean oil | 3.353 | 1.878 | 2.3795 |
| Mineral/vitamin premix $^{\Omega}$ (0.3%) | 3.000 | 3.000 | 4.000 |
| Common salt | 2.981 | 3.224 | 3.212 |
| DL – methionine | 1.414 | 1.537 | 1.703 |
| Inert | 0.600 | 0.600 | - |
| Metabolizable energy (kcal/kg) | 2,900.00 | 2,860.00 | 2,900.00 |
| Crude protein (g/kg) | 195.00 | 180.0 | 180.0 |
| Available phosphorus (g/kg) | 10.00 | 4.60 | 4.50 |
| Calcium (g/kg) | 4.50 | 11.00 | 4.00 |
| Sodium (g/kg) | 1.70 | 1.80 | 1.80 |
| Digestible lysine (g/kg) | 8.563 | 7.495 | 8.083 |
| Digestible methionine+ cystine (g/kg) | 6.754 | 6.513 | 7.098 |
| Digestible methionine (g/kg) | 4.000 | 3.931 | 4.606 |
| Digestible threonine (g/kg) | 6.177 | 5.564 | 5.795 |
| Digestible tryptophan (g/kg) | 1.929 | 1.728 | 1.724 |

$^{\Omega}$ Warranty levels: Iron (min) 16.67 g/kg, copper (min) 3,333. 00 mg/kg, manganese (min) 26.67 g/kg, zinc (min) 20.00 g/kg, iodine (min) 333.00 mg/kg, cobalt (min) 67.00 mg/kg, selenium (min) 160.00 mg/kg, Retinol (min) 1920.00 mg/kg, Cholecalciferol (min) 21.67 mg/kg, Tocopherol (min). 12,120.91 mg/kg, Menadione sodium (min) 1,066.00 mg/kg, Thiamine (min) 1,066.00 mg/kg, Riboflavin (min) 2,666.00 mg/kg, niacin (min) 16.00 g/kg, pantothenic acid (min) 6,400.00 g/kg, Pyridoxine (min) 1,600.00 mg/kg, folic acid (min) 533.34 mg/kg, biotin (min) 32.00 mg/kg, Cyanocobalamin (min) 10,670.00 mcg/kg, hill (min) 200.00 g/kg.

Inert: Washed Sand (0.600)

Replaced the inert: Halquinol (0.050); Organic Acids (OA): 0.300; Symbiotic (S): 0.300; Essential oil (EO): 0.300; OA (0.300) + S (0.300); EO (0.300) + S (0.300).

The tibias were weighed and placed in a forced ventilation oven at 105 °C for 72 h, then they were weighed again and crushed with a mortar and pistil. The ground samples were identified to determine mineral matter (MM) according to the methodology described by Silva & Queiroz (2002).

## Statistical analysis

The data were processed and analyzed in five consecutive steps described below:

1st Step (*Simple effect*): The data were subjected to analysis of variance (ANOVA) followed by SNK means test ($p<0.05$) for all variables according to the following mathematical model:

Yij = μ + βi + εij,

In which: Yij = observation j of the experimental unit submitted to treatments i, μ = general constant, βi = effects of treatments and εij = random error associated with each observation.

2nd Step (*Variable reduction*): Exploratory Factor Analysis (EFA) was performed using indicators to reduce the number of variables (36) into new variables (11). Factor analysis is an interdependence technique used to reduce a large set of variables into factors or indicators (Hair *et al.*, 2009). The factor analysis model is expressed by Eq. (1):

$$X_1 = a_{11} \times F_1 + a_{12} \times F_2 + \ldots + a_{1m} \times F_m + e_p$$
$$X_1 = a_{11} \times F_1 + a_{12} \times F_2 + \ldots + a_{1m} \times F_m + e_p$$
$$\ldots \qquad \text{Equation (1)}$$
$$X_1 = a_{11} \times F_1 + a_{12} \times F_2 + \ldots + a_{1m} \times F_m + e_p$$

where is the p$^{th}$ score of the standardized variable (p = 1, 2, …, m), is the extracted factor, is the factor loading, and is the error.

Factor scores for each group were estimated by multiplying standardized variables by the coefficient of the corresponding factor score, as follows Eq. (2)

$$F_1 = d_{11} \times X_1 + d_{12} \times X_2 + \ldots + d_{1j} \times X_{jp}$$
$$F_2 = d_{21} \times X_2 + d_{21} \times X_2 + \ldots + d_{2j} \times X_{jp}$$
$$\ldots \qquad \text{Equation (2)}$$
$$F_j = d_{p1} \times X_1 + a_{j1} \times X_2 + \ldots + d_{jp} \times X_{jp}$$

In which: is the j-th factor extracted, is the factor score coefficient, and p is the number of variables (Hair *et al.*, 2009). The selection of variables for inclusion in the analysis was determined by the commonality values with a minimum acceptable value of > 0.50 (Figueiredo Filho & Silva Júnior, 2010). Principal components (PC) with eigenvalues greater than 1 were used (Kaiser rule; Kaiser, 1960). The rotated component matrix was developed using orthogonal rotation, varimax method, with the Kaiser normalization method. The PCs were renamed based on eigenvectors with values greater than +0.50 and less than -0.50, and then used as a new variable in the canonical discriminating analysis ($C_{DA}$; Step 4) and hierarchical cluster analysis ($H_{CA}$; Step 5). The assumptions, the partial and total variance of the eigenvalues, the eigenvectors of the variables and the commonality of EFA are presented in Table 3.

3rd Step (*Differentiation of treatments*): CDA was carried out to discriminate and verify if there is a dynamic classification of birds in their group of origin. The general model of the CDA is described in the Eq (4)

$$Z_n = \alpha + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_n X_n \qquad \text{Equation (3)}$$

In which: is the dependent variable (treatments), is the intercept, are the explanatory variables, and is the discriminant coefficients for each explanatory variable.

The $C_{DA}$ was performed by the simultaneous method, which considers the inclusion of all variables that are important in the multivariate model.

4th Step (*Group dynamics and selection of variables*): $H_{CA}$ was performed to verify the dynamics of the clusters according to the dietary inclusion or not of growth promoters. The model used for hierarchical clustering is described in Eq. (3).

$$d[k,(i,j)] = max\,[d(k,i), d(k,j)] \qquad \text{Equation (4)}$$

**Table 3 –** Principal components selected in the factor analysis, assumption, eigenvalues, partial and cumulative variances and correlation coefficients of the variables under study.

| Principal components | Assumptions: KMO | Assumptions: Bartlett's test (*p* – valor) | Eigenvalues, % partial variance, (% cumulative variance) | Variables | Correlation coefficients | Commonality |
|---|---|---|---|---|---|---|
| Performance | 0.55 | < 0.0001 | 2.604<br>65.11<br>(65.11) | Weight gain | 0.96 | 0.99 |
| | | | | Feed conversation | - 0.88 | 0.99 |
| | | | | Final weight | 0.96 | 0.99 |
| Feed intake | | | 1.38<br>34.39<br>(99.50) | Feed Intake | 0.66 | 0.99 |
| Precocity | 0.56 | < 0.05 | 1.607<br>40.17<br>(40.17) | Age at first egg | 0.71 | 0.51 |
| | | | | 1st egg weight | 0.72 | 0.53 |
| | | | | 100% production age | 0.76 | 0.68 |
| Production age | | | 1.06<br>26.53<br>(66.71) | 50% production age | 0.97 | 0.94 |
| Sexual maturity | 0.64 | < 0.0001 | 2.21<br>55.27<br>(55.27) | Crest height | 0.93 | 0.86 |
| | | | | Crest length | 0.91 | 0.83 |
| | | | | Oviduct | 0.73 | 0.70 |
| Carcass development | | | 1.122<br>28.06<br>(83.33) | Sternum length | 0.98 | 0.95 |
| Bone characteristics | 0.50 | = 0.01 | 1.48<br>49.63%<br>(49.63%) | Deformity | 0.86 | 0.74 |
| | | | | Mineral matter | 0.86 | 0.75 |
| Bone resistence | | | 1.002<br>33.39<br>(83.01%) | Resistance | 0.99 | 0.99 |
| Tractogastrointestinal | 0.53 | = 0.002 | 1.752<br>35.04<br>(35.04%) | Proventricle | 0.88 | 0.77 |
| | | | | Intestines | 0.87 | 0.77 |
| Glands | | | 1.191<br>23.82<br>(58.87) | Liver | 0.59 | 0.81 |
| | | | | Pancreas | 0.90 | 0.87 |
| Bowel morphometry | | | 1.13<br>22.58<br>(81.45) | Intestines legth | 0.89 | 0.86 |

This agglomerative algorithm calculates the shortest distance between elements i and j using the distance matrix dij (Hair *et al.,* 2009). Ward's method (Ward, 1963) was used with Euclidean distance as a similarity measure in $H_{CA}$. Individual eigenvectors of the birds were used by treatments using the regression method to generate the matrix. From these, the average factor score per treatment was generated and for each factor renamed in Step 3. As a complementary analysis, the result of the grouping was plotted using a heatmap generated using the HeatMapper software program (Babicki *et al.,* 2016).

5th Step (*Relationship between variables*): EFA was performed again, but in this step in order to identify the relationship of performance, biometrics and sexual maturity simultaneously. The Kaiser-Meyer-Olkin (KMO) criterion was equal to 0.575 and Bartlett's sphericity test was significant ($p$<0.001). The three principal components were extracted by orthogonal rotation (varimax method) for better interpretability and plotted on a three-dimensional graph. Finally, the correspondence analysis (animal observations and vector direction) was performed to verify the dynamics of bird clusters according to whether or not they included growth promoters, and to define which variables stood out according to treatments.

# RESULTS

Performance (Table 4), relative weight of digestive organs and length of intestine (Table 5), relative weight of ovary, oviduct, height and length of ridges and sternum length (Table 6), bone quality (Table 7) and sexual maturity (Table 8) were similar ($p$>0.05).

It was found that 73.80% of the birds were correctly classified in their group of origin (Figure 1) with no treatment with 100% accuracy in classifying the birds. Although the first two canonical functions discriminated against 58.55%, there was no discriminatory power ($F_1$: $P$ = 0.09 and $F_2$: = $P$ = 0.251), thus indicating a similarity between treatments when we analyzed them together. The heat map combined with cluster analysis with dietary treatments with or without inclusion of growth promoters and the eleven factors extracted is shown in Figure 2. A uniform static pattern was generally verified (neutral values and low values positive/negative Z-scores) between the extracted factors and the treatments. The formation of the following three horizontal groups formed by the treatments was also observed: 1) negative control, symbiotic and organic acids + symbiotic; 2) positive control and essential oil and 3) essential oil + symbiotic and organic acids. Bone characteristics had a negative correlation with organic and symbiotic acids + carvacrol, while the other treatments showed a weak positive

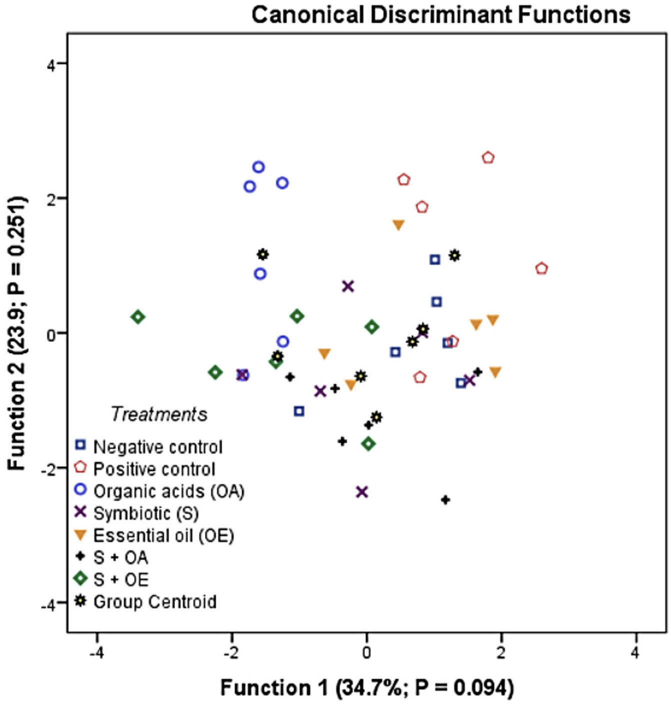


**Figure 1** – Biplot of the canonical discriminant analysis evaluating the performance, biometry of digestive and reproductive organs, bone characteristics and sexual maturity of light replacement pullets at the 8 to 17-week-old stage.

Note: Each point represents a bird.

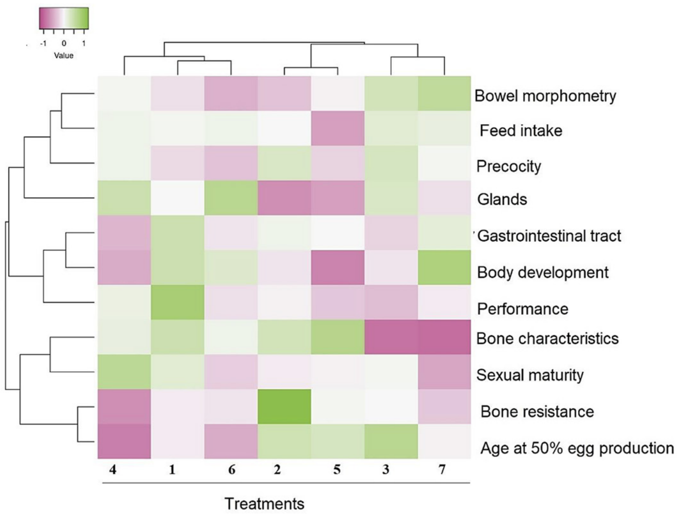


**Figure 2** – Dendrogram conjugated in a heat map of the principal components versus dietary inclusion treatments or not of growth promoters in light replacement pullets at the 8 to 17-week-old phase.

Note: Positive (dark green) and negative (dark pink) values of Zscores show the positive and negative association between identified factors (lines) and treatments (columns).

Neutral values (clear or white) of Zscores show a low association between identified factors (rows) and treatments (columns).

1 - Negative control, 2 - Positive control, 3 - Organic acids, 4 - Symbiotics, 5 - Essential oil, 6 - Organic acids + Symbiotics, 7 - Symbiotics + Essential oil

correlation. The organs had a negative correlation with the positive control, and the production age at 50% had a negative correlation with the symbiotic, while the other treatments showed a weak positive correlation.

The relationship between performance, biometrics and sexual maturity of pullets according to growth promoters is found in Figure 3. No group dynamics (Fig. 3A) were observed between treatments. The birds showed a similar response among the growth promoters under study. It was also found that the first three components explained 60.55% of the variation in the data (Fig. 3B), in which the first component revealed an association of weight gain, weight and feed consumption with the length of the sternum, while the second component showed an association of the maturity characteristics with the conversion. Finally, the third component indicated that the morphometry of the crest is positively related to the reproductive system of the birds.

## DISCUSSION

In this study we investigated $N_{GP}$ to replace conventional growth promoters used in the diet of replacement pullets using a systematic approach with the combination of different multivariate analysis techniques, in addition to pointing out predictive variables for maturity, performance and precocity in the poultry sector.

The use of natural additives did not influence the performance of the pullets (Table 4), which is a desirable effect, since the additives and their associations enabled maintaining the beneficial flora of the digestive tract without interfering in the use of nutrients in the diet, and if so, excluding the deleterious effects of such additives. On the other hand, the absence of effects in the negative control diet (without additives) on the performance variables (an unexpected fact) is evidence that these birds probably did not suffer any field challenge which could harm them, and with this favorable condition being extended in other diets. Such results were not expected, given that all pullets underwent routine management of the breeding and rearing phases when they were manipulated and subjected to the beak treatment, which are sometimes stressful moments that could make them more susceptible to pathogenic microorganisms present in the environment, thus triggering lower performance, but which was not observed. In a study by Stefanello *et al.* (2020), in which organic acids (fumaric, sorbic, malic and citric acid) and essential oils (thymol, vanillin and eugenol) were used, the results showed that the association of $O_A$ and $E_O$ resulted in greater

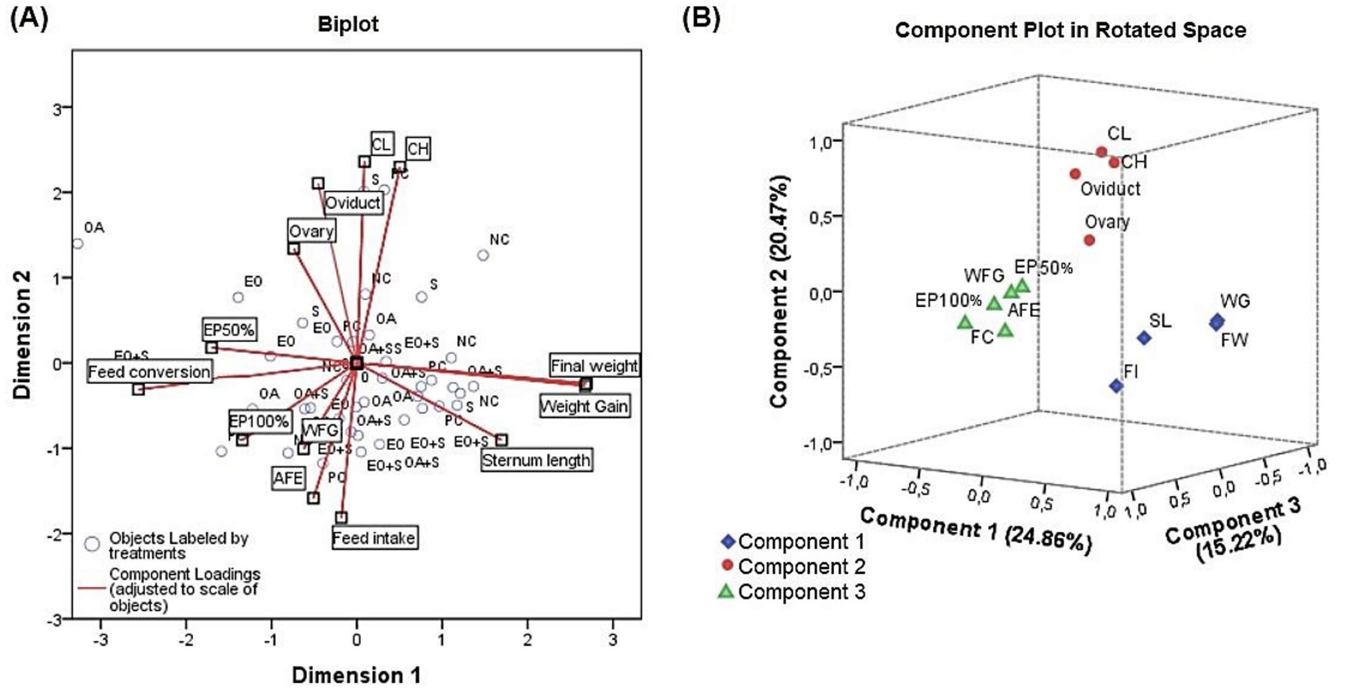


**Figure 3** – Biplot of the dimension analysis (A) and exploratory factor analysis (B) evaluating the relationship of performance, biometrics and sexual maturity according to the natural growth promoters with the feeding of light replacement pullets.

Note: $N_C$, Negative control, $P_C$, Positive control, $O_A$, Organic acids, S, Symbiotics, $E_O$, Essential oil, $A_O$ + S, Organic acids + Symbiotics, $E_O$ + S, Essential oil + Symbiotics. EP50%, age 50% of egg production (weeks), EP100%, age 100% of egg production (weeks), $S_L$, sternum length (mm), $W_G$, weight gain (g/ave), $F_W$, Final weight (g/ave), $F_I$, feed intake (kg/ave), $F_C$, feed conversion (g/g), $A_{FE}$, age at first egg (weeks), $W_{FG}$, final weight gain (g/ave), $C_L$, crest length (mm), $C_H$, crest height (mm).

**Table 4 –** Performance of light replacement pullets fed with growth promoters in the 8 to 17-week-old phase.

| Treatments | Weight (g/bird) | | Weight gain (g/bird) | Feed intake (g/bird) | Feed conversion (g/g) |
|---|---|---|---|---|---|
| | 8 weeks | 17 weeks | | | |
| Negative control | 652.6 | 1126.7 | 474.5 | 3182.3 | 6.00 |
| Positive control | 653.4 | 1118.9 | 465.5 | 3055.8 | 6.61 |
| Organic Acids (OA) | 653.7 | 1121.8 | 467.9 | 3106.8 | 7.00 |
| Symbiotic (S) | 654.2 | 1128.6 | 474.4 | 3086.6 | 6.53 |
| Essential oil (EO) | 654.2 | 1090.2 | 436.0 | 3096.2 | 7.10 |
| OA + S | 654.2 | 1113.6 | 459.4 | 3070.5 | 6.74 |
| EO + S | 653.9 | 1147.3 | 494.3 | 3087.5 | 6.35 |
| Mean | 653.7 | 1121 | 467.4 | 3095.9 | 6.61 |
| Coefficient of variation (%) | 1.00 | 4.35 | 10.53 | 6.71 | 10.40 |
| *p*-value | 0.999 | 0.653 | 0.638 | 0.973 | 0.149 |

performance of cutting broilers due to the greater apparent digestibility of nutrients and ileal energy. The authors attributed these results to the protective effect of the additives on the intestinal mucosa, reducing mucosa colonization by pathogenic microorganisms and the production of toxins.

A biometric analysis of digestive organs enables identifying possible changes arising from the nutritional management to which the birds were subjected, as the development of these organs is directly related to the animal's feed (Farias *et al.,* 2019). Considering the use of $N_{GP}$ in the feed of pullets, changes in digestive organs could show harmful effects of such ingredients, therefore making their use in birds' diets unfeasible (Li *et al.,* 2018). However, no effect on the biometrics of digestive organs was found (Table 5), so it can be inferred that the natural growth promoters can be used as additives in the nutrition of birds. Such information is relevant, considering that the pullets were in the growth phase, where the cellular renewal of the digestive tract is extremely important for nutrient absorption from the diets (Adedokun & Olojede, 2019).

Pullets are currently known for their high productivity; however, good development in the breeding and rearing phases is necessary in order to optimize performance in adulthood (D´Agostini *et al.,* 2017). In the present study, none of the natural growth promoters resulted in changes in the relative weights of ovary and oviduct or in the development of ridges and sternum.

The integrity of the intestinal mucosa is extremely necessary for nutrient absorption which compose the skeletal structure of birds (Sugiharto, 2016). Such a structure is responsible for supporting the muscular development of pullets, supporting the body weight acquired by them, which is one of the best performance indicators for a future laying hen (Hy Line, 2020). Thus, any imbalance of the intestinal microbiota caused by an ineffective growth promoter could hinder the nutrient absorption essential for bone mineralization, causing changes in the bone characteristics of the birds' tibias, which was not observed.

Current replacement pullets are precocious and nutritionally more demanding, and the results obtained at both the beginning and throughout the entire production phase are dependent on the proper management and nutrition carried out in the breeding and rearing periods (Névoa *et al.,* 2013). The age at the first egg is considered a good indicator of the birds' precocity and it is necessary that the pullets have adequate body reserves at the end of

**Table 5 –** Relative weight of digestive organs and intestine length of replacement pullets fed diets containing growth promoters in the 8 to 17-week-old phase (average of 6 repetitions with 5 birds each).

| Treatments | Proventricle (%) | Gizzard (%) | Liver (%) | Pancreas (%) | Intestines (%) | Intestine length (cm) |
|---|---|---|---|---|---|---|
| Negative control | 0.42 | 2.24 | 1.99 | 0.23 | 2.22 | 118.60 |
| Positive control | 0.43 | 2.13 | 1.78 | 0.22 | 2.11 | 110.00 |
| Organic Acids (OA) | 0.39 | 2.01 | 1.91 | 0.26 | 2.28 | 118.20 |
| Symbiotic (S) | 0.41 | 1.96 | 1.85 | 0.24 | 2.04 | 115.40 |
| Essential oil (EO) | 0.42 | 2.25 | 1.80 | 0.22 | 2.11 | 114.40 |
| AO + S | 0.42 | 2.30 | 1.94 | 0.26 | 1.95 | 120.75 |
| EO + S | 0.43 | 2.22 | 1.81 | 0.24 | 2.15 | 121.20 |
| Mean | 0.42 | 2.16 | 1.87 | 0.24 | 2.12 | 116.82 |
| Coefficient of variation (%) | 12.50 | 11.07 | 9.90 | 15.70 | 8.80 | 6.14 |
| *p*-value | 0.881 | 0.275 | 0.517 | 0.564 | 0.251 | 0.224 |

**Table 6 –** Relative weight of the reproductive organs, height and length of the crests and sternum length of replacement pullets fed diets containing growth promoters in the 8 to 17-week-old phase (average of 6 repetitions with 5 birds each).

| Treatments | Ovary (%) | Oviduct (%) | Crest height (mm) | Crest length (mm) | Sternum length (mm) |
|---|---|---|---|---|---|
| Negative control | 0.07 | 0.11 | 18.24 | 36.58 | 121.06 |
| Positive control | 0.05 | 0.15 | 17.87 | 32.49 | 119.27 |
| Organic Acids (OA) | 0.07 | 0.11 | 17.77 | 35.78 | 118.63 |
| Symbiotic (S) | 0.08 | 0.18 | 19.24 | 36.61 | 117.29 |
| Essential oil (EO) | 0.09 | 0.12 | 19.84 | 38.05 | 115.67 |
| OA + S | 0.07 | 0.06 | 16.05 | 35.71 | 120.14 |
| EO + S | 0.06 | 0.05 | 15.45 | 32.27 | 122.68 |
| Mean | 0.07 | 0.11 | 17.72 | 35.23 | 119.25 |
| Coefficient of variation (%) | 17.22 | 32.24 | 23.39 | 15.31 | 3.41 |
| *p*-value | 0.527 | 0.439 | 0.662 | 0.608 | 0.168 |

**Table 7 –** Bone characteristics of replacement pullets fed diets containing growth promoters in the 8 to 17-week-old phase (average of six repetitions with five birds each).

| Treatments | Seedor index (mg/mm) | Resistance (kgf/cm²) | Deformity (mm) | Mineral matter (g/kg) |
|---|---|---|---|---|
| Negative control | 2.03 | 9.70 | 5.16 | 45.50 |
| Positive control | 2.24 | 10.64 | 4.93 | 46.52 |
| Organic Acids (OA) | 2.17 | 9.81 | 3.96 | 43.86 |
| Symbiotic (S) | 2.42 | 9.74 | 4.57 | 46.48 |
| Essential oil (EO) | 2.02 | 9.91 | 4.88 | 47.95 |
| OA + S | 2.17 | 9.75 | 4.30 | 47.10 |
| EO + S | 2.51 | 9.53 | 4.08 | 44.21 |
| Mean | 2.22 | 9.86 | 4.55 | 45.94 |
| Coefficient of variation (%) | 15.19 | 9.89 | 14.48 | 5.34 |
| *p*-value | 0.245 | 0.764 | 0.078 | 0.122 |

the rearing period to withstand the physical wear and tear arising from the beginning of the laying phase, and in turn be able to maintain the production rate and egg quality throughout the production phase (D´Agostini *et al.,* 2017). The Lohmann lineage manual indicates the start of poultry production at 19 weeks of age, however we observed that the birds started laying in a similar way between the 18$^{th}$ or 19$^{th}$ weeks (Table 8), i.e. the pullets showed standard development according to recommendations of the lineage. Therefore, it is inferred that the growth promoters used were efficient in promoting intestinal quality, favoring the absorption of nutrients and ensuring the performance found. In the case of the age of birds with 50% production, the same manual recommends the interval between 20 to 21.43 weeks as a standard result. All treatments are within this range (Table 8), with the exception of the birds in the treatment that contained symbiotic without association (19.86 weeks), which shows the early and good development of the birds. It is noteworthy that the results obtained with natural additives are equal to conventional growth promoters, thus demonstrating their equivalence of use (viability of use) in promoting the precociousness of pullets.

**Table 8 –** Sexual maturity of replacement pullets fed diets containing growth promoters at the stage of 17 to 28 weeks of age.

| Treatments | Age (weeks) | | | 1$^{st}$ egg weight (g) |
|---|---|---|---|---|
| | 1$^{st}$ egg | 50 % | 100 % | |
| Negative control | 18.67 | 20.17 | 22.67 | 40.00 |
| Positive control | 19.17 | 20.67 | 22.50 | 41,67 |
| Organic Acids (OA) | 18.66 | 20.66 | 23.00 | 42.83 |
| Symbiotic (S) | 19.00 | 19.83 | 22.17 | 41.33 |
| Essential oil (EO) | 19.00 | 20.50 | 22.50 | 40,25 |
| OA + S | 19.00 | 20.00 | 22.16 | 40,00 |
| EO + S | 18.67 | 20.33 | 22.17 | 42.83 |
| Mean | 18.88 | 20.31 | 22.45 | 41.39 |
| Coefficient of variation (%) | 3.21 | 2.78 | 4.69 | 5.81 |
| *p*-value | 0.64 | 0.094 | 0.777 | 0.230 |

The Lohmann lineage manual does not specifically mention the age of the birds with 100% production, possibly because it is a recommendation for producing birds on an industrial scale, and individually observing the production of the cages would hinder the logistics of egg management. However, the same age was observed between treatments, inferring that the alternative growth promoters used with conventional promoters were efficient in promoting the intestinal quality necessary for the absorption of nutrients from the diets. The strain recommendation for the average egg weight in the first week of production (19 weeks) is 40.7g (Lohmann LSL Lite, 2016). The weight of the first egg per treatment was specifically evaluated in this study, and this difference in methodology probably explains the fact that the weight of the first egg in the negative control treatments, essential oils, and organic acids with symbiotic have weights below 40.7g. However, there was no statistical difference between treatments for this variable, thus not disqualifying any of the alternative natural growth promoters and their associations.

The non-discriminatory power of the two discriminant functions (Fig. 1) together with the static pattern of the clusters plotted on the heat map (Fig. 2) confirmed the similarity in the physiological, morphological and productive responses, in addition to identifying the cluster dynamics of these responses and the mechanisms of action of additives. The centroid position of the treatments in the CDA biplot corresponds to the treatments in the clusters. The formation of the three horizontal clusters (Figure 2): 1) symbiotic, negative control and OA + S; 2) positive control and EO; and 3) organic acids and S+OE are probably justified by the similarity in their mode of action. Symbiotic and organic acids are similar in reducing pH, maintaining a healthy microbial balance in the gut and promoting intestinal integrity and immune health, selectively reducing colonization by pathogenic microorganisms such as *E. coli,* Salmonella and *C. perfringens* (Funari Junior *et al.,* 2011; Jha Rajesh *et al.,* 2020; Stefanello *et al.,* 2020). The similarity of the positive control (antibiotic) with the essential oil is due to the effect that both have in limiting pathogenic bacteria in the intestinal lumen, increasing some beneficial microorganisms to the intestinal microflora (Toledo *et al.,* 2007; Fernandes *et al.,* 2014), and finally the association of organic and symbiotic acids + essential oil is due to the beneficial effects that these additives have in protecting the intestinal mucosa, which is responsible for the digestion and absorption processes, preventing microbial infections, acting in the bacterial cell, preventing its growth and multiplication (Zardo *et al.,* 2015; Stefanello *et al.,* 2020).

We also observed a neutral relationship in the vertical clusters in the dendrogram between the variables and the treatments, except for: bone strength, which presented a positive correlation with the positive control; this is due to the effect that antibiotics have in improving the use of nutrients in the diets and consequently promoting an improvement in weight gain and feed conversion rate (Fernandes *et al.,* 2014; Torres *et al.,* 2015).

Some negative correlations which occurred may be due to some conditions, such as handling, imposed sanitary challenge, quality of the feed provided, concentrations of additives used in the diet, or even the heterogeneity of the intestinal microflora; these all contribute to these results (Dalolio *et al.,* 2015; Araujo *et al.,* 2019), since the use of these additives improved performance, nutrient digestibility, egg production and quality and prevent microbial infections, as cited by some studies (Tang *et al.,* 2017; Wang *et al.,* 2019; Stefanello *et al.,* 2020).

The grouping between the performance characteristics is related to the gastrointestinal tract development and animal development, which in turn is due to the balance of the beneficial intestinal microbiota which provide an adequate environment for the digestion and absorption processes of the diet nutrients, helping to maintain the intestinal mucosa. The relationship between sexual maturity, bone strength and age at 50% production highlights the body and skeletal development of the pullets, while the uniformity of the flock in the breeding and rearing stages and sexual maturity at the appropriate age are crucial factors in the production of replacement pullets and directly interfere in the productivity of future laying hens (Bain *et al.,* 2016).

In this study, the potential for egg production and precocity of birds was assessed through factor analysis and correspondence, in which the characteristics were linearly distributed and associated with each other by means of their respective loads and vectors, making it possible to compose factors with physiological, morphological and productive interpretation. The homogeneous pattern presented in the classification of birds (Fig. 3) is justified by the similar response of the inclusion that the additives, isolated or not, provide in the diets, especially in the performance of the intestinal microflora, since the additives in studies act in modulating the microbiota and in the immunomodulatory responses, thereby increasing the performance and productivity and decreasing mortality in farm animals (Dalólio *et al.,* 2015; Thelma *et al.,* 2019).

The association of weight gain, bird weight, feed consumption and sternum length (Fig. 3B) is due to the fact that if feed consumption is increasing, the birds will consequently present greater weight, weight gain and greater sternum length. This result demonstrates that the development of the sternum bone (housing the pectoral muscle) is of substantial importance for the production period of replacement birds, i.e. laying hens with good development of the pectoral muscle are more capable of maintaining high production as adults.

The age at the first egg constitutes very important data to characterize sexual maturity, which can be influenced by the age and body composition of the birds (Braz *et al.,* 2011). This probably explains the relationship that occurred in sexual maturity between age at 50 % and 100% of production, age at first egg and feed conversion. The association of oviduct, ovary and ridge length and height in the third factor can be explained by the fact that the larger the ridge, the better the development of the ovary and oviduct, meaning that the ridge is related to the maturation of the reproductive organs of birds.

Finally, this study is a pilot project focused on final responses of poultry production, however future studies evaluating the inclusion of the same natural growth promoters, with conventional promoters in the diet should be carried out with a focus on biochemical, hematological, immunological and microbiological responses in order to better understand the physiological mechanisms of growth promoters in light replacement pullets.

## CONCLUSION

Organic, symbiotic acids and essential oils can replace traditional growth promoters in pullet diets. Crest height and length are predictive variables for the development of reproductive organs, while egg production and weight are related to feed conversion. A systematic approach adopting multivariate techniques with different objectives is essential to answer and understand the relationships between the variables.

## DECLARATION OF ANIMAL RIGHTS

All clinical procedures and examines were approved by the Ethical Committee Use Animals from State University of Acaraú Valley (Protocolo N° 002.05.019. UVA.504.03).

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## CONFLICT OF INTERESTS

The authors state no conflic of interest.

## CONSENT TO PUBLISH

The authors state that all human participants in the research provided consent for the publication of the data.

## DATA AVAILABILITY

Data will be made available on request

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## AUTHORSHIP CONTRIBUTION STATEMENT

Myrianlene Moura Castro – Conceptualization, Data collect, Writing - original draf and Final review. Silvana Cavalcante Bastos Leite – Supervision, Project administration, Data curation, Data analysis, Methodology, Validation, Writing - original draft, Funding acquisition and Final review. Rogervânia Silva de Farias – Conceptualization, Validation; Final review. Cláudia Goulart de Abreu - Conceptualization, Validation, Final review. Carla Nágila Cordeiro – Conceptualization, Validation, Final review. Ednardo Rodrigues Freitas – Conceptualization, Validation, Final review. Robson Mateus Freitas Silveira – Data analysis, Methodology, Data curation, Conceptualization, and Final review.

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