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Association Between Productive Parameters and Intestinal Histomorphological Findings in Broilers Supplemented with Probiotics (*Saccharomyces cerevisiae*, *Lactobacillus acidophilus* and *Bacillus subtilis*)

Asociación entre Parámetros Productivos y Hallazgos Histomorfológicos en Pollos de Engorde Suplementados con Probióticos (*Saccharomyces cerevisiae*, *Lactobacillus acidophilus* y *Bacillus subtilis*)

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SUMMARY: This study evaluates the effect of probiotics *Saccharomyces cerevisiae*, *Lactobacillus acidophilus* and *Bacillus subtilis* on production parameters and intestinal histomorphology of broilers of 45 days of age. Eleven 45-day-old Ross 500 broilers were used and classified as control group (CG) (n = 5) or supplemented with probiotics group (n = 8). Histopathological evaluation of duodenum, ileum, and jejunum was performed. The area of the villi height, base and apex were evaluated as well as the size and number of crypts. In addition, mucus production was quantified in different portions of the small intestine. The villi present duodenum of broilers supplemented with probiotics had a greater area (p = 0.0127), a greater basal width (p = 0.0049) and a greater apical width (p = 0.0024), as well as a greater crypt area (p = 0.0189). Significantly higher levels of mucus were noted in the duodenum (p = 0.0480) and jejunum (p = 0.0480) of broilers supplemented with probiotics. We suggest that probiotic supplementation improve the intestinal nutrients absorption.

KEY WORDS: Poultry; Probiotic; Intestinal; Histomorphology.

Abbreviations: CG: Control group, HPF: High power field, H & E: Hematoxilin and eosine, PAS: Periodic acid Schiff, PG: Probiotic group.

INTRODUCTION

The poultry industry greatly supports the world food security, providing energy, protein, and micronutrients essential for humans, with short production cycles (Food and Agriculture Organization of the United Nations & World Health Organization, 2006).

The use of antimicrobials as growth promoters in the poultry industry has greatly decreased due to increasing reports of antimicrobial resistance and the use of alternatives such as probiotics have increased in return (Olnood *et al*., 2015; Wu *et al*., 2016; Callens *et al*., 2018). Probiotics are selected live microorganisms that when administered in adequate amounts, allow for eubiosis, including promotion of healthy intestinal microbiota, and intestinal integrity, guaranteeing the timely use of dietary nutrients (Olnood *et al*.).

Probiotics work through competitive exclusion, stimulation of the immune system, acting as an antioxidant, maintaining the integrity of the intestinal mucosa, stimulating the production of the intestinal mucus layer, and hindering the reproduction and colonization of pathogenic bacteria's (Olnood *et al*.; Liong, 2015; Ambalam *et al*., 2016).

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The beneficial effects of probiotics include the production of secreted factors which promote cytokine production and cell-mediated immunity. Probiotics stimulate appetite and improve the intestinal balance, shortening the time required to stabilize the microflora (Liong; Ambalam *et al*.). Probiotics produce toxic compounds that inhibit the growth of pathogenic microorganisms, improve feed conversion, and promote growth (Ambalam *et al*.). They have been reported as factors that contribute to the prevention of cancer, cholesterol, and triglycerides reduction 10–12, since they increase lipid catabolism and decrease lipid absorption (Luo *et al*., 2013). This characteristic ensures prolonged probiotic transit time in the gastrointestinal tract which causes cholesterol lowering effects in vivo (Cavallini *et al*., 2011). Probiotics bind to cholesterol through their cell membrane, which reduces the amount of intestinal cholesterol available for absorption (Ishimwe *et al*., 2015).

Lactic acid producing bacteria and yeasts are the microorganisms most used as probiotics in animal production (Kuebutornye *et al*., 2019). Among the most used species of probiotics are lactobacilli such as *Lactobacillus acidophilus*, bacilli such as *Bacillus subtilis*, and yeasts such as *Saccharomyces cerevisiae* (Souza *et al*., 2017; Fernandez-Pacheco *et al*., 2018). *Bacillus subtilis* has been shown to improve performance in the first weeks of life by increasing the total digestibility of dry matter, crude protein, and apparent metabolic energy, and increasing the pH of the intestinal content. *Lactobacillus acidophilus* has been shown to improve performance in high temperatures and increase fatty acids in meat (Jahromi *et al*., 2016).

Saccharomyces cerevisiae increased the size of the crypt area in duodenum and jejunum, and increased mucus production in the duodenum, which by increasing the intestinal absorption surface could surely result in improvements in the productive parameters (Quevedo *et al*., 2020). Likewise, the effects of the probiotic *Saccharomyces cerevisiae boulardii* were evaluated in broilers exposed to campylobacteriosis through the oral administration of 105 CFU / ml of *Campylobacter jejuni*. In response of this stimulus the histological and morphometric analysis revealed greater length of villi and depth of the crypts. Therefore, the yeast improved the intestinal microbiota and accordingly it was obtained a better feed conversion rate and weight gain; in addition to the protective effect of the probiotic against Campylobacter (Massacci *et al*., 2019). Previous studies report that probiotic supplementation increases the height and width of intestinal villus as well as the depth of the intestinal crypt compared to groups not supplemented with probiotics (Liong; Zarei *et al*., 2017; Forte *et al*., 2018; Quevedo *et al*.). Crypt is considered the villi factory. A deeper crypt may indicate a faster tissue turnover allowing a renewal of villi that have suffered alterations due to pathogenic actions and consequently better nutritional absorption capacity (Korn *et al*., 2007). This results in greater absorption of nutrients (Massacci *et al*.), with better feed conversion rate and weight gain (de Lemos *et al*., 2013). A greater depth in the crypts is reflected in more mucin production (Quevedo *et al*.) and therefore an effective response of physical protection against pathogens (Gao *et al*., 2008).

The mucus that lines the surface of the gastrointestinal mucosa plays an important role as a protective barrier, preventing viral adhesion, and absorption of pathogenic bacteria and toxins through the intestinal epithelium. The presence of mucus in the intestine is altered by additives in the diet, physiological status, and subsequent development of the chicken. Broilers supplemented with a commercial product containing *Saccharomyces cerevisiae*, 15 days after having administered the treatment revealed significantly higher densities and sizes of goblet cells for broilers supplemented with yeast in relation to their respective controls (Quevedo *et al*.).

Few studies in chickens investigated the synergic effect of probiotics in intestinal histomorphometry where the height, basal width, apical width and areas of the villi and the crypt are evaluated in the different sections of the small intestine. The aim of this study was to examine changes in intestinal morphology resulting from probiotic supplementation. We evaluated the intestinal histomorphology in broilers supplemented with *Saccharomyces cerevisiae*, *Lactobacillus acidophilus*, *Bacillus subtilis* to determine their effect on production parameters and intestinal histomorphology at 45 days of age.

MATERIAL AND METHOD

Tissues samples were obtained from 11 broilers chicken, breed Cobb 500, 45 days old. Individuals were distributed in two experimental groups, reared in the same shed, divided into cages as control and experimental group. The individuals had water and food at will; they were fed with commercial Italcol® initiation diet during the first 15 days according to the manufacturer's instructions and fattening. Supplementation with probiotic began from day 15th with 5 days of accustoming, and starting records from day 20 of its consumption, were supplemented with *Saccharomyces cerevisiae*, *Lactobacillus acidophilus* and *Bacillus subtilis*, assuring concentration of 107cfu/g/day experimental diet.

The broilers were part of the project: "Physiological and productive behavior in broilers supplemented with probiotics" (Gutiérrez-Castro & Corredor-Matus, 2017), approved by the ethics committee of the Universidad de los Llanos (Number of register: FCARN-15-2014). All the data related to productive parameters with productive performance were obtained from this project. Such as daily feed intake, weight gained and feed conversion for 30 days, boneless breast production and boneless leg-leg ratio with respect to the carcass.

The individuals were distributed in two experimental groups: control group (CG) (n = 5) and supplemented with probiotic (PG) (n = 8). Formalin-fixed samples from 11 chickens were obtained. The tissues were processed according to routine histotechnic protocol were stained with hematoxylin and eosin (H & E), and periodic acid Schiff (PAS).

Production parameters evaluation. Within the productive parameters were included: feed consumption, weight gain and carcass yield, measured by calculating the proportion of boneless breast and the leg-to-thigh ratio (leg-to-thigh) with the carcass.

Histomorphological evaluation. Intestinal tissues were analyzed using a Leica DM 500 light microscope (Leica Microsystems) and photomicrographs for analysis were taken using a Leica ICC50W camera. Parameters evaluated include epithelial and crypt hyperplasia, villi orientation, crypt-intestinal villi rate, villi areas, crypt areas, number of cells with mitotic activity and number of goblet cells.

The count of epithelial number cells in villi was performed in 40x, counting the nuclei of the epithelial cells from the base to the apex of 5 intestinal villi per section of the ID of each animal of the experimental group. The number of crypts was counted in 5 high-power fields (HPF) per intestinal segment of each individual. The orientation of the villi was determined by everyone classified as longitudinal or in Zigzag.

To establish the crypt-intestinal villi rate, the length of the villus was calculated by measuring the distance between the tip of the villus and its base, excluding the crypt. This procedure was done in each of the experimental groups. For each intestinal section five measurements were made, an average was calculated, and the area of the villi was calculated according to the following expression:

Villi area = (Basal width + apical width) x height of the villus

In order to measure the area of the crypt, the transverse and longitudinal diameter of the crypt was determined and then the relation between the villus areas versus the crypt was calculated using the Image Pro Plus 5.0 software (Media Cybernetics, Silver Spring, MD, USA). The percentage occupied by goblet cells marked with PAS staining in each intestinal segment per individual was determined in 5 photographed fields. To determine the number of cells with mitotic activity, manual counting was performed in 5 high power fields for the intestinal segments of each individual.

Statistical analysis. All statistical analysis was done using ANOVA, Newman-Keuls Multiple Comparison Test, Kruskall Wallis, Mann Whitney and Pearson's correlation coefficient were calculated. All statistical procedures were performed with GraphPad Instat software, version 5.01. Statistical difference was considered with a $P<0.05$.

Ethics Statement. All experiments were conducted in compliance with protocols reviewed and approved by the bioethics committee of the Universidad de los Llanos. This work was developed in the city of Villavicencio, Colombia, in the Universidad de los Llanos, Histopathology Laboratory of the School of Animal Sciences and Veterinary Medicine Program, located at 420 masl, average temperature of 28 ° C, annual precipitation of 4050 mm and average relative humidity of 85 %.

RESULTS

Production parameters. The production parameters, feed consumption, weight gain and carcass yield, measured by calculating the proportion of boneless breast and the leg-to-thigh ratio (leg-to-thigh) at the time of slaughter are shown in Table I. No significant differences were found in any of the variables analyzed, although the table shows that there was a better behavior in each one of these in PG, especially the productive performance, where proportion of boneless breast and proportion leg-to-thigh was higher in PG.

Number of Cells per villi. The duodenum of the PG (2679 ± 236.6) had a significantly higher ($p = 0.0039$) numbers of epithelial cells per villus compared to CG (1569 ± 37.05). The jejunum of the PG (1720 ± 74.98) had a significantly higher ($p = 0.0018$) numbers of epithelial cells per villus compared to CG (1112 ± 146.8). No significant differences were observed in the ileum (Fig. 1).

Number of Crypts of intestinal gland (Lieberkühn). The number of Lieberkühn crypts per millimeter in the duodenum was significantly higher ($p = 0.0121$) in the CG (14.35 ± 1.799) compared to the PG (9.841 ± 0.5970). No statistical

Table I. Effect of probiotics on production parameters, in 45-day-old broilers.

Feed intake (g)		Weight gain (g)		Food conversion		Boneless breast meat (carcass %)		Leg:thighs. (carcass %)	
CG	PG	CG	PG	CG	PG	CG	PG	CG	PG
128.7	130.18	61.49	62.61	1.97	2.045	24.04	28.39	24.48	26.41

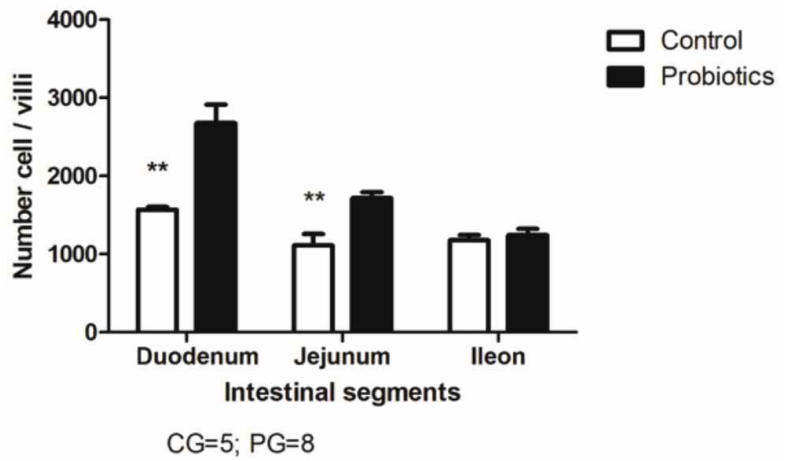

Fig. 1. Number of cells per villi of the intestinal segments evaluated: duodenum, jejunum, and ileum, in individuals of the group supplemented with probiotics (PG) and control group (CG). There was statistical difference in the number of cells per villi in duodenum and jejunum.

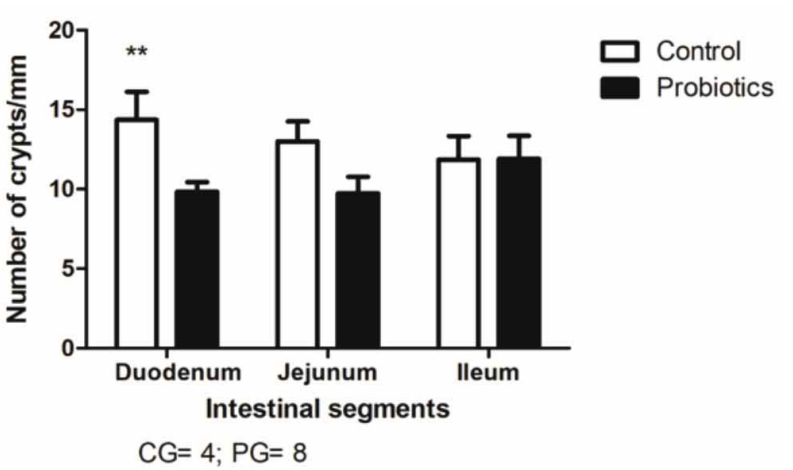

Fig. 2. Number of Lieberkühn crypts per millimeter in the intestinal segments evaluated: duodenum, jejunum, and ileum of individuals from the group supplemented with probiotics (PG) and control group (CG). Statistical difference was observed in duodenum.

difference was observed in jejunum (p = 0.0707) or ileum (p = 0.9811) between PG and CG (Fig. 2).

Villus orientation. No significant differences were observed in the orientation (longitudinal or zig-zag) of the intestinal villi of CG and PG. However, PG showed tendency to have villi zigzag orientation versus CG (Figs. 3A,B).

Villi Width and Height. The duodenum of broilers in the PG had significantly higher basal width (p = 0.0049) (155.4 mm ± 13.90 mm) compared to the duodenum of broilers in the CG (103.9 mm ± 8.74 mm), as well as higher apical width (p = 0.0024) (90.62 mm ± 5.205 mm) vs control (61.13 mm ± 4.49 mm). A higher area in PG (p = 0.0127) (368700 mm^2 ± 47170 mm^2) vs control (200300 mm^2 ± 10910 mm^2) was observed, being more robust the duodenal intestinal villi of PG compared to CG. While in jejunum and ileum these differences were not observed. There was not statistical difference regarding the height of the villi versus to the intestinal segments between the two groups (Fig. 4).

Villi and Crypt area. The duodenal villi area of broilers in the PG (368700 mm^2 ± 47170 mm^2) was significantly greater (p = 0.0127) than the villi area of broilers in the CG (200300 mm^2 ± 10910 mm^2).

The duodenal Lieberkühn crypts area of broilers in the PG (3239 mm^2 ± 209.9 mm^2) was significantly greater (p = 0.0189) than the duodenal Lieberkühn crypts area of broilers in the CG (2140 mm^2 ± 340.2 mm^2).

In the jejunum (p = 0.0068) (3738 mm^2 ± 350.5 mm^2) PG vs (2243 mm^2 ± 202.4 mm^2) CG (Fig. 5).

Mitotic activity. The PG presented a higher number of mitotic figures vs. CG. In the sections of the duodenum (p = 0.0003) (5.575 ± 0.3494) PG vs (2.800 ± 0.3847) CG. In jejunum (p = 0.0012) (4.850 ± 0.3354) PG vs (2.600) ± 0.3795) CG, and in ileum (p = 0.0046) (4.725 ± 0.3564) PG vs (2.840 ± 0.3487) CG (Fig. 6).

Mucus production. Statistical difference was evidenced (p = 0.0480) in the percentage of mucus production in the duodenum and jejunum, in 5 HPF. The probiotic group had a higher proportion of mucus in the duodenum (p = 0.0480) (14.48 % ± 1.64 %) PG vs (8.67 % ± 0.97 %) CG. Also, in jejunum (p = 0.0480) (18.02 % ± 1.48 %) PG vs (11.50 % ± 1.68 %) CG (Fig. 7). While in the ileum and cecum there were no significant statistical differences.

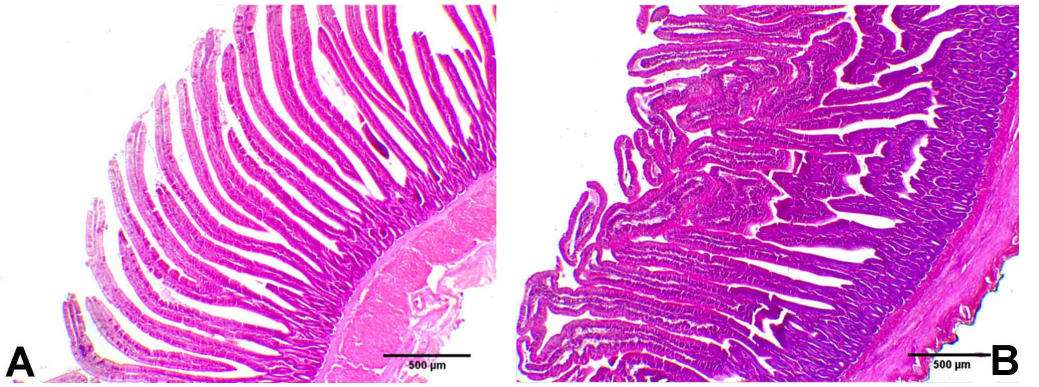

Fig. 3. Duodenum longitudinal section. (A) The villi of the control group duodenum show linear orientation (H&E, 10x). (B) The villi were much more robust and those of adjacent sites intersect, in the group treated with probiotics that show a zigzag orientation (H&E, 10x).

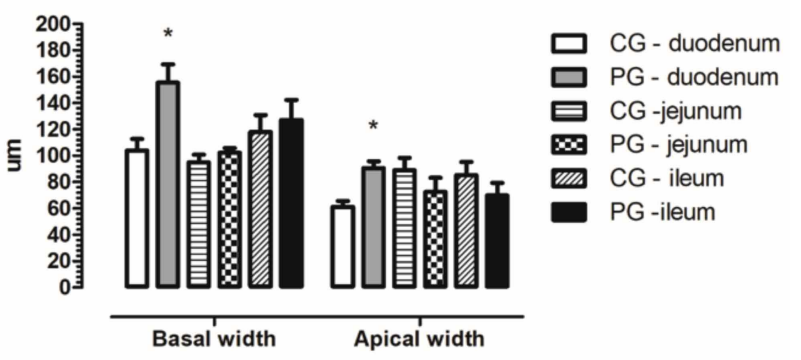

Fig. 4. Basal and apical width (um) in the intestinal segments evaluated: duodenum, jejunum, and ileum of individuals from the group supplemented with probiotics (PG) and control group (CG). A statistical difference was observed in duodenum, where width and height were greater.

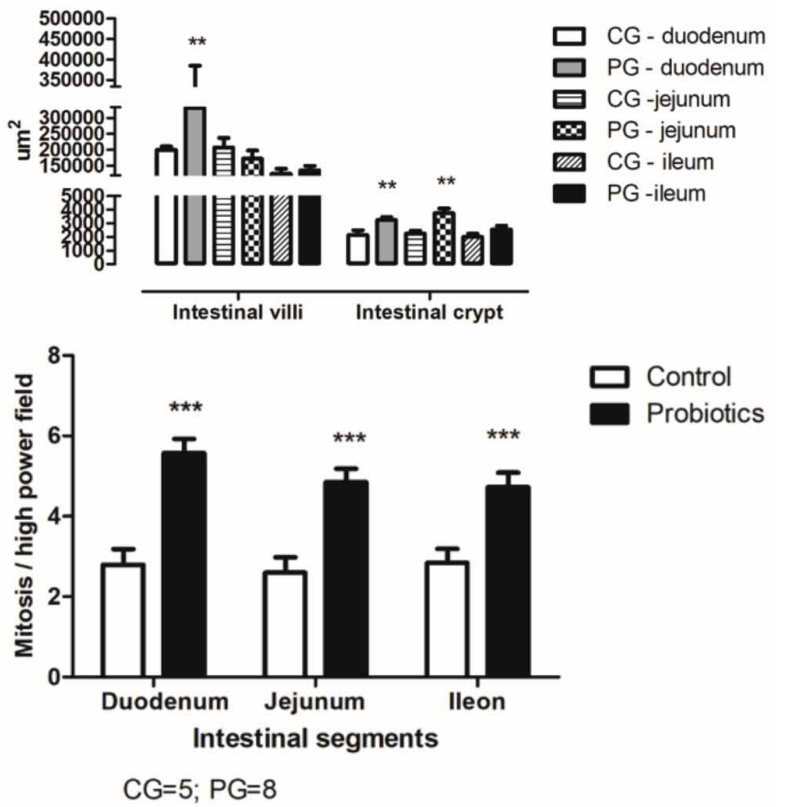

Fig. 5. Crypt area (µm) in the different intestinal segments evaluated in probiotic group (PG) vs. control group (CG).

Fig. 6. Number of mitotic figures in the crypts of the intestinal segments evaluated (HPF): duodenum, jejunum, and ileum of individuals from the group supplemented with probiotics (PG) and control group (CG).

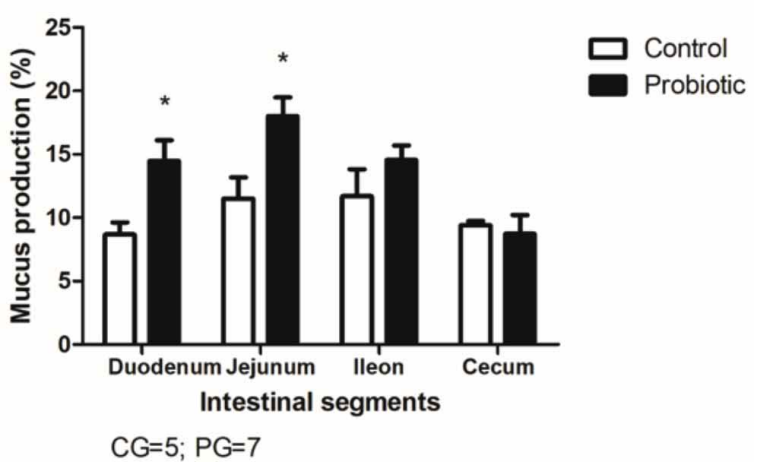

Fig. 7. Percentage of mucus production of the intestinal segments evaluated (HPF): duodenum, jejunum, and ileum of individuals from the group supplemented with probiotics (PG) and control group (CG). Statistical difference was observed in duodenum and jejunum, with higher mucus evidenced.

Correlation Productive parameters and histomorphology variables. A significant positive correlation was found between the leg-to-thigh ratio and the crypt area variables at the ileum (Table II). A significant negative correlation was observed between the GP and mucus production in the ileum (Table II). In addition, leg: thigh and CA ratio showed R = 0.8593 with p = 0.0283.

Table II. Correlations between production parameters and histological variables in the three intestinal sections in broilers supplemented with probiotics.

Production parameter	Intestinal section	Histological feature	R	P
Feed consumption	Duodenum	Cells in mitosis	0.8413	0.0358
Feed consumption	Jejunum	Crypt area	0.8184	0.0465
Feed consumption	Jejunum	Cells in mitosis	0.8991	0.0147
Weight gain	Ileum	Mucus production	-0.9134	0.0109
Boneless breast portion	Ileum	Crypt transverse diameter	0.8464	0.0336
Leg:thigh percentage	Jejunum	Villi area	-0.8200	0.0457
Leg:thigh percentage	Jejunum	Crypt area	0.9875	0.0002
Crypt area	Ileum	Mucus production	0,7830	0.0147
Leg-thigh percentage	Jejunum	Crypt longitudinal diameter	0,8157	0,0478

R, Correlation index. P, p- value

DISCUSSION

A higher number of mitotic figures were observed in the three sections of the small intestine in PG, compared to the small intestine sections of the CG. Previous studies have shown that a single dose (Samanya & Yamauchi, 2002) or multiple doses (Ghosia *et al*, 2011) of probiotics stimulate cell proliferation in the duodenum, jejunum and ileum. Probiotics increase cell proliferation by reducing ammonia production, which has deleterious effects in the intestine (Beski & Al-Sardary, 2015). Besides, probiotics stimulate the production of short-chain fatty acids, such as butyric acid, which works as a source of energy for cells in the intestine and stimulate cell proliferation of the intestinal epithelium (Liong).

In this study, a positive relationship was found between feed consumption and the number of mitotic figures. However, no significant differences in the daily feed consumption were observed between groups. These results suggest that having a greater number of enterocytes and cells in mitosis could ameliorate the digestive and absorption capacity. Thus, subjects treated with probiotics have a more efficient gut function.

In contrast, studies have found decrease (Altaher *et al*., 2015) or increase in food consumption during probiotic supplementation. The greater food consumption has been related with enhance of growth performance in poultry due to improvement of the microflora in the digestive system. Moreover, it has been associated to increase in gene expression of the orexigenic hormone ghrelin, and improvement of the weight gain due to anabolic effect of ghrelin. This hormone stimulates the release of growth hormone increasing muscle mass (Yada *et al*., 2008).

A positive correlation was found between crypt area and food consumption in duodenum and jejunum, as previously reported (Ghosia *et al*.). The crypt area of these sections was significantly higher in PG than in CG. An increase in the crypt area suggests a higher mitotic activity in the crypt to maintain adequate cell turnover in the epithelium (Luquetti *et al*., 2012).

Higher nutrient availability resulting from a rise in food intake may stimulate intestinal activity. Amino acids are absorbed in the jejunum and duodenum, while carbohydrates are absorbed exclusively in the jejunum. Therefore, a greater crypt area in those intestinal segments may have contributed to the correlation with the leg-to-thigh ratio due to a higher nutrient absorption.

Besides, the above suggests that, by increasing the cell proliferation in the jejunum, which is in turn, an indication of the activity of the villus, there is the possibility that a decrease occurs in the physiological activity of the ileum, due to the lower arrival of nutrients to this region, which could determine the decrease in AC.

The number of crypts was significantly lower in the duodenum in PG, although the crypt area was significantly higher in duodenum and jejunum in this group. We suggest that having a larger area of crypta in the PG, there may be a decrease in its total number, without this representing a lower intestinal secretion or decrease in digestive function or absorption, according to the production parameters.

Number of cells per villi was higher in the duodenum and jejunum in PG. In Arbor Acres chicks, an increase of cells per villi has been observed between 4- and 10-days post-hatching in the duodenum and jejunum. Studies in turkeys show an increase in the cells per villi of the duodenum and jejunum (Approximately 800 and 650 cells/villi respectively) in contrast to the ileum (Approximately 200 cells/villi) at 2 weeks post-hatching (Uni *et al*., 1999). This increase in the cells per villi of the duodenum and jejunum may be an effect of a higher number of mitotic figures found in these intestinal segments.

In this study was found that PG had a greater basal and apical width, and a greater area of the villus and crypt at the duodenum compared with chickens in the CG. Findings previously reported with the addition of *Saccharomyces cerevisiae* to the diet (Arce *et al*., 2008) have been associated with increased cell turnover (Liong; Allahdo *et al*., 2018). As well as the villi area, the villi orientation can influence the nutrients absorption. Considering that villi from the PG have a zigzag orientation, we infer that this type of orientation increases the time of permanence of the intestinal content, improving digestibility and absorption (Yamauchi & Isshiki, 2007; Nicoletti *et al*., 2010; Barrera-Barrera *et al*., 2014). In PG, weight gain had a significative inverse correlation with mucus production in the ileum. Where was found a significant increase in the number of mitotic figures in the crypt, as in the other sectors of the intestine. This explains the rise in the mucus production.

Previous studies have described increase in the population density of goblet cells, an increase in the production of mucins in the small intestine, a thickening of the mucus layer associated with an increase in diet probiotics, as well as improving the innate defense against pathogens from the diet (Mack *et al*., 2003; Tsirtsikos *et al*., 2012).

However, the negative relationship can be explained by the fact that in the ileum, the amount of nutrients that arrive for the chemical digestive process is low, since the previous sectors have done most of the work. Therefore, the contribution of nutrients absorbed from this sector is lower, so that it will participate in a minimal way in the PG of the broilers. Similarly, an increase in mucus production, without the accompaniment of digestive enzymes (proteases and amylases) that perform terminal digestion, could obstruct the absorption of the final nutrients that remain in the intestinal lumen, a situation that would contribute to the negative relation found.

CONCLUSION

This study concluded that the addition of probiotics in the diet of broilers has several effects on intestinal histomorphology, which can be quantified. Including increase in basal and apical width of the villi of the duodenum, increase the crypt area, villi orientation, among others. Which was reflected in differences in productive parameters and very surely in different processes of nutrient and metabolic absorption.

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RESUMEN: Este estudio evalúa el efecto del uso de probióticos como: *Saccharomyces cerevisiae*, *Lactobacillus acidophilus*, *Bacillus subtilis* en parámetros productivos e histomorfologia intestinal de pollos de engorde de 45 días de edad. Fueron usados 11, los cuales fueron clasificados en grupo control (CG) (n = 5) y grupo suplementado con probióticos (PG) (n = 8). Fue realizado análisis histopatológico de secciones de duodeno, íleon y yeyuno. Fue evaluado ancho, altura y área del ápice de la vellosidad, área y número de criptas. Además, fue estimada la producción de moco en los diferentes segmentos del intestino delgado. Fue observada mayor área de la vellosidad en duodeno, PG (p = 0.0127), ancho basal mayor en PG (p = 0.0049) ancho apical mayor en PG (p = 0.0024), así como mayor área de criptas en PG (p = 0.0189). No fueron encontradas diferencias significativas respecto a los segmentos de yeyuno e íleon. PG presentó mayor producción de moco en duodeno (p = 0.0480) y en yeyuno (p = 0.0480). Concluimos que la suplementación con probióticos en pollos de engorde genera cambios en la histomorfologia intestinal, evidenciables en áreas apicales y basales de las vellosidades intestinales. Soporte financiero: Dirección General de Investigaciones – Universidad de los Llanos.

PALABRAS CLAVE: Pollos de engorde; Probióticos; Intestinal; Histomorfologia.

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