Comparison effect of lasalocid, diclazuril, probiotic and synbiotic on histomorphological changes of small intestine induced by **E. tenella**

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Keywords
Coccidiosis,
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Probiotic,
Synbiotic,
Anticoccidial drugs.

Summary
This study aimed to investigate the comparison of effect of anticoccidal drugs including lasalocid and diclazuril with probiotic and synbiotic on the growth performance and intestinal morphology in broiler chicken. One hundred eighty chickens (Ross 308, 1 day old) were randomly divided into 6 equal groups (n=30) including the negative control (basal diet), the positive control (basal diet+oral inoculation of 3×104 sporulated oocytes of *E. tenella*, and four treatment groups. At days of 28 and 49 of age, 9 chickens were blindly chosen from each group were scarified by decapitation and their various segments of small intestine including ileum, jejunum, and duodenum were evaluated histomorphologically. We found that the economic losses resulted from coccidial infection in the poultry industry are caused by the decreased performance of broiler chicken induced by morphological changes in the any three segments specially jejunum. The anticoccidial drugs, synbiotic and probiotic can partially prevent morphological changes in any three segments of small intestine in broiler chicken with coccidiosis. Since morphological changes in the jejunum begin earlier than in other parts and surface area of jejunal villi is important for nutrition absorbance as well as growth performance, lasolacid was found to a be more efficient treatment in this regard**.**

Introduction

Avian coccidiosis is one the most common parasitic diseases in poultry with significant economic implications (Peek and Landman 2011). Coccidiosis causes intestinal necrosis leading to insufficient absorption and increases of mortality rate. As a result, it causes substantial economic in the poultry industry (Abdelrahman *et al.* 2014; Ott *et al.* 2018). The global economic losses of coccidiosis poultry industry is estimated to exceed US$ 3 billion annually (Noack *et al.* 2019). Despite the presence of control programs and effective anticoccidial drugs in poultry, satisfactory control and treatment of coccidiosis remains difficult (Chapman 2009). In addition, resistance to the all available chemicals drugs as well as the public concerns over potential drug residues in poultry products have put restrictions on the use of certain agents (Peek and Landman 2011). By the time clinical signs become apparent in poultry, it is often too late, making prophylaxis the preferred method to control the disease (Chapman 2009). Diclazuril is one of the most common anticcocidal drugs that has been demonstrated to induce ultrastructural changes in merozoites and to disrupt the transmembrane potential of mitochondria (Zhou *et al.* 2010). Lasalocid is another anticcoccidal drug that can reduce oocyst production in naturally infected animal and in a few cases improves growth performance and decrease clinical sign (Noack *et al.* 2019). Synbiotics are defined as the combination of probiotics and prebiotics. Probiotics consist of live microorganisms, which may have beneficial effects on health of the host when administered in adequate amounts. Probiotic bacteria have been used to enhance animal performance, possibly by

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maintaining the normal microflora of host animals (Hassanpour *et al*., 2013). The primary action of probiotics is to reinforce the intestinal mucosal barrier against adverse agents (Fioramonti *et al*., 2003). Probiotic bacteria also stimulate antigen-specific and nonspecific immune responses. Ingestisting probiotics results in the reduction of some fecal enzymes, which are capable of converting pro-carcinogens to carcinogens in the gastrointestinal system (Shah, 2007). Prebiotics, on the other hand, are defined as 'on-digestible feed ingredients that beneficially affect the host by selectively stimulating the growth or activity or both of limited number of bacteria in the colon, which can improve host health (Hassanpour *et al*., 2013). The probiotics have been administrated as an alternative to anticoccidial drugs either via feed additives or drinking water (Dalloul *et al*. 2005). **Use of a probiotic approach** provide protection and decrease the adverse effects of the Eimerian infection (El-Sawah *et al*. 2020). Recent studies have shown that synbiotics can improve the performance of broiler chickens and in increases the caecal beneficial bacteria (Mookiah *et al*. 2014). It has been recently shown that the **anticoccidial** drugs such as diclazuril and lasalocid in combination with probiotics improve the growth performance of broiler chickens. Therefore, this study aimed to investigate the comparison of effect of anticoccidial drugs including lasalocid and diclazuril with probiotic and synbiotic on growth performance and intestinal morphology in broiler chicken.

Materials and methods

One hundred eighty chickens (*Ross 308,* 1 day old) were randomly divided into 6 equal groups (n=30) including the negative control (receiving basal diet), the positive control (receiving basal diet + oral inoculation of 3×10^4 sporulated oocytes of *E. tenella*, and four treatment groups. All four treatment groups were fed basal diet + oral inoculation 3×10^4 sporulated oocytes *of E. tenella* from 14 day of age to end of study. The treatment groups received one of following protocols: 500g/1000 kg of diet/daily lasalocid, 1kg/1000 kg of diet/daily synbiotic, 1kg/1000 kg of diet/daily diclazuril, and 1kg/1000 kg of diet/daily probiotics (commercially is known **SafMannan)**. To evaluate production indexes, all chicken and their food consumption were weekly weighted and food conversion ratio was calculated for each group. A same and standard condition such as ventilation, humidity, light cycle, temperature, and vaccination for rearing period of 49 days with free access to water and diet was applied for all groups. The basal diet for all chicken was equally formulated based on corn and soybean. At days of 28 and 49 of age, 9 chickens were **randomly** selected from each group were sacrified by decapitation and their various segments of small intestine including ileum, jejunum, and duodenum were firstly seized and fixed in Clark fixative, a solution containing 25% acetic acid and 75% ethyl alcohol, for 45 min. For longer storage, the segments were placed in 50% ethyl alcohol. **Each gastrointestinal tract section** was cut into multiple segments along its length and then stained using periodic acid Schiff (PAS) reagent for 3 to 5 min. The segments were washed twice in PBS each of 5 min and located on the paraffin within the petri dish. The rows of villi were cut using cataract knife along with their length so that the **villi** were adhered and located inside to each other. The sections were transferred over the glass slides, covered with cover-slip and finally analyzed by using a Motic microscope analyzer with eyepiece graticules (10×) at magnification of ×100. Five villi and five crypts were randomly chosen tomeasure the height of the villi as well as area and depth of the crypts. The villi heights and width were determined from top of villi to top of lamina properia, and distance between two bases of villi, respectively. The depth of lieberkuhn crypt (the epithelia of the villi extending down into the lamina propria where they form crypts) was measured from base of villus to the lowest part of crypt where the epithelial layer of each hemi-cryptcontinues with the epithelium of the villus. Surface area was calculated using the formula = (π) × (VW) × (VL) in which VW= villus width and VL= villus length as previously reported by Sakamoto *et al.* (2000)(Sakamoto *et al.* 2000). The livers and spleens were also removed and **weighed**.

On days 28 and 49 of age, 9 chicks from each group were **sacrificed** by decapitation for evaluation of their intestinal morphometric variables including height, width, and surface area of villus as well as depth of crypts in the various segments of duodenum, jejunum and ileum as previously described by Zamani Moghaddam *et al*. (2009)(Zamani Moghaddam *et al.* 2009).

Statistical analysis

The obtained data were analyzed using SPSS software version 20. All data are presented as mean ± standard deviation (SD). Data were considered to be statistically significant when p < 0.05 by one way ANOVA, followed by Tukey HSD test.

Results

The altered intestinal morphology resulting from the various treatment groups as well as the control group are presented in Figures 1 to 6.

Duodenal morphology findings on day 28 of age

No significant difference was seen amongst all the groups in term of the duodenal villus height and width (Fig. 1a, 1b). However, a significant decrease in depth of duodenal lieberkuhn crypts was indentified in the lasalocid and diclazuril groups compared to **the other groups** (P<0.05)(Fig. 1c). The duodenal villus surface area was significantly lower in the diclazuril and the probiotic groups than those of other groups (P<0.05) (Fig. 1d).

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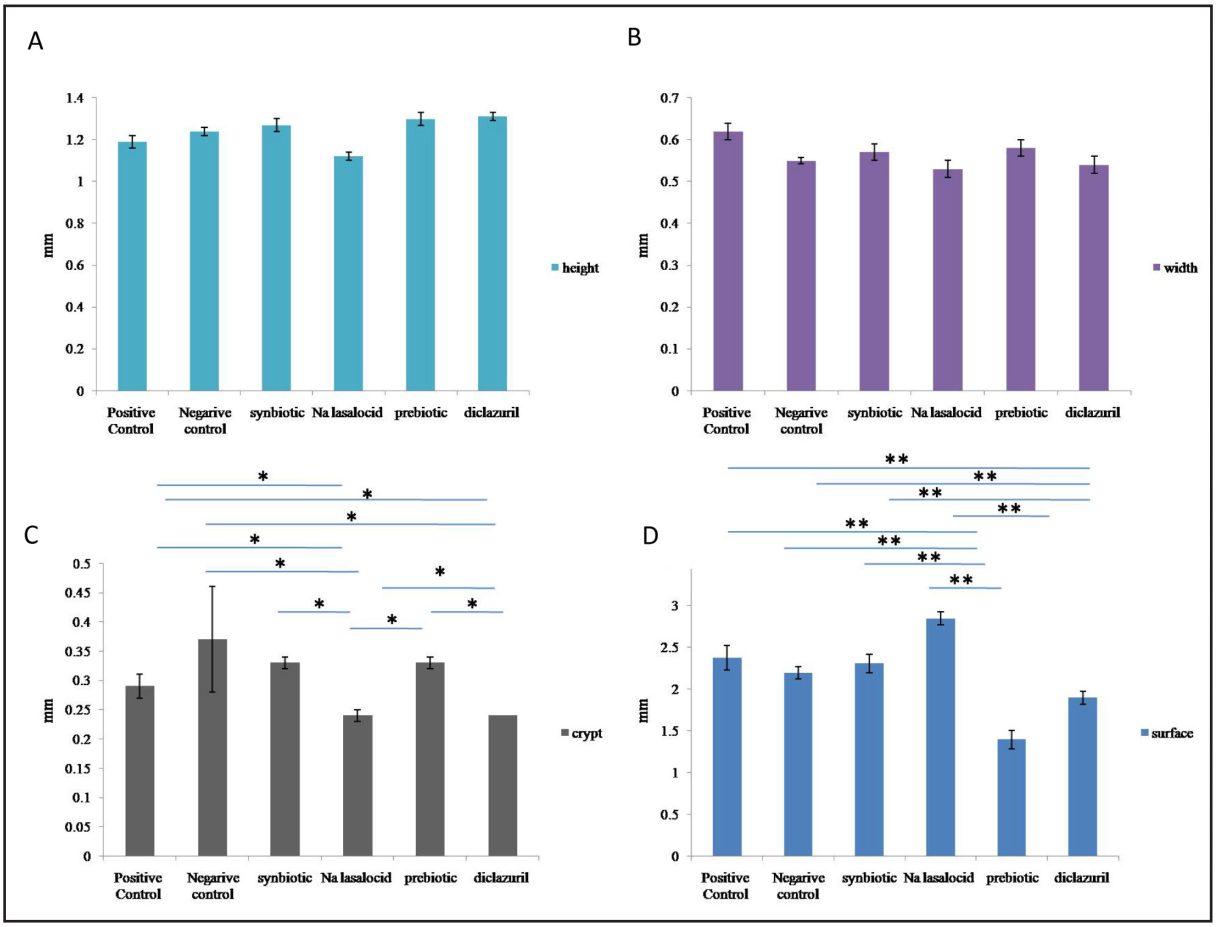

Figure 1. *Duodenal morphometric variables including height, width, and surface area of villus as well as depth of crypts on 28 day of age in various groups ** indicating P<0.001, * indicating P<0.01.*

Duodenal morphology findings on day 49 of age

The negative control group showed significantly higher duodenal villus height than those of other groups (P<0.05)(Fig .2a). A significant decrease in the duodenal villus height was seen in the probiotic and the diclazuril compared to negative, synbiotic and lasalocid groups (P<0.05) (Fig.2a). The negative control and synbiotic groups showed significantly lower duodenal villus width than those of other groups (P<0.05) (Fig .2b). The negative control and probiotic groups showed significantly lower duodenal depth of crypt than those of other groups (P<0.05) (Fig .2c). The negative control group showed significantly lower duodenal surface area of villus than those of other groups on day 49 of age (P<0.05) (Fig .2d). In addition, the lower duodenal surface area of villus was seen in the synbiotic group that those of the positive control and diclazuril groups.

Jejunum morphology findings on day 28 of age

A significant decrease in height, width, and depth of crypt surface area of jejunum villi was identified in the positive control group compared to all other groups, as well as in the all treatment group compared to the negative control group (P <0.05) (Fig. 3a, 3b, 3c, 3d). A significant increase in the depth of crypt was seen in the diclazuril group compared to other treatment group (P<0.05) (Fig. 3d).

Jejunum morphology findings on day 49 of age

The negative control group showed significantly higher jejunum villi width than those of other groups (P<0.05)(Fig. 4a). There was no significant difference between the positive control group with all treatment groups in term of jejunum villi width (P>0.05) (Fig. 4a). The negative control group showed significantly increase in the depth of crypt compared to other groups (P<0.05)(Fig. 4b). A significant decrease in the positive control, synbiotic and lasalocid groups was seen compared to the probiotic and diclazuril groups in term of depth of crypt.

A significant decrease in the diclazuril group was also detected compared to the probiotic respected to depth of crypt (P<0.05)(Fig. 4b).

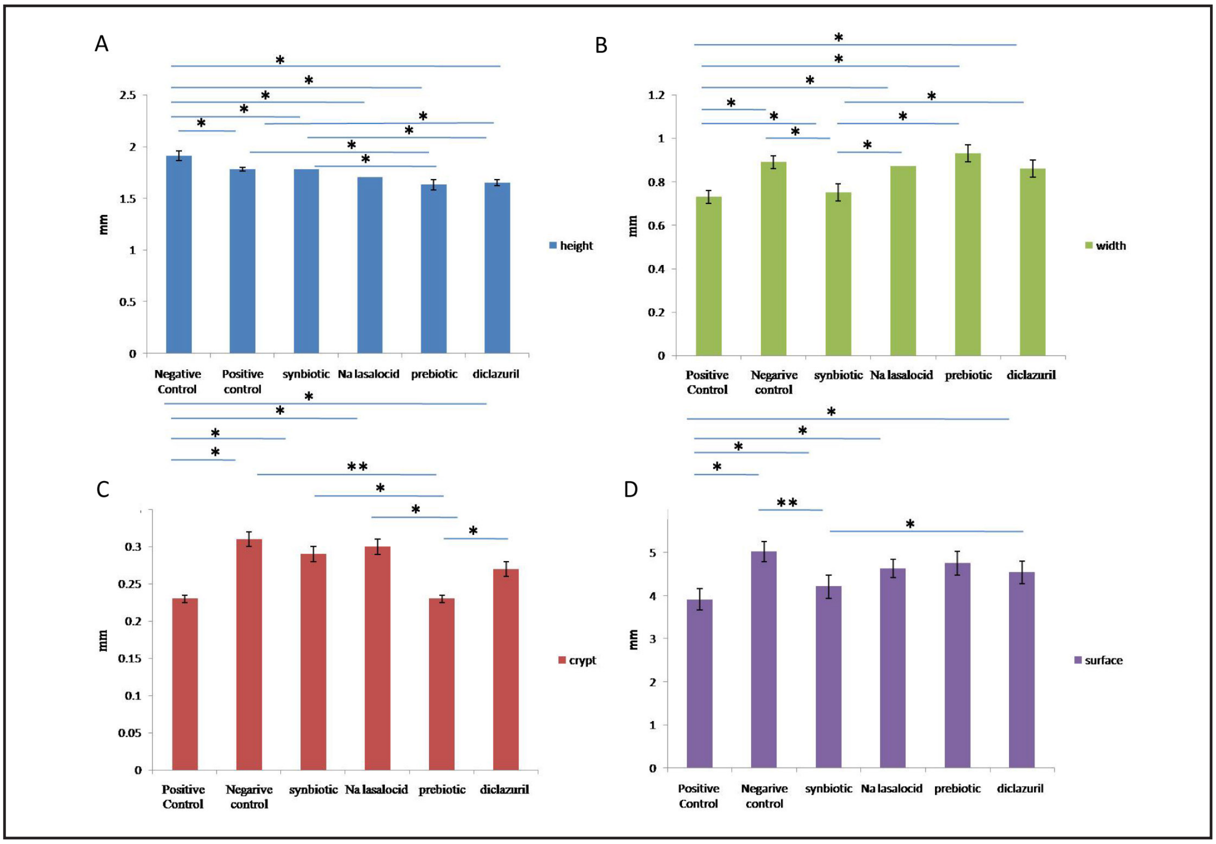

Figure 2. *Duodenal morphometric variables including height, width, and surface area of villus as well as depth of crypts on 49 day of age in various groups ** indicating P<0.001, * indicating P<0.01.*

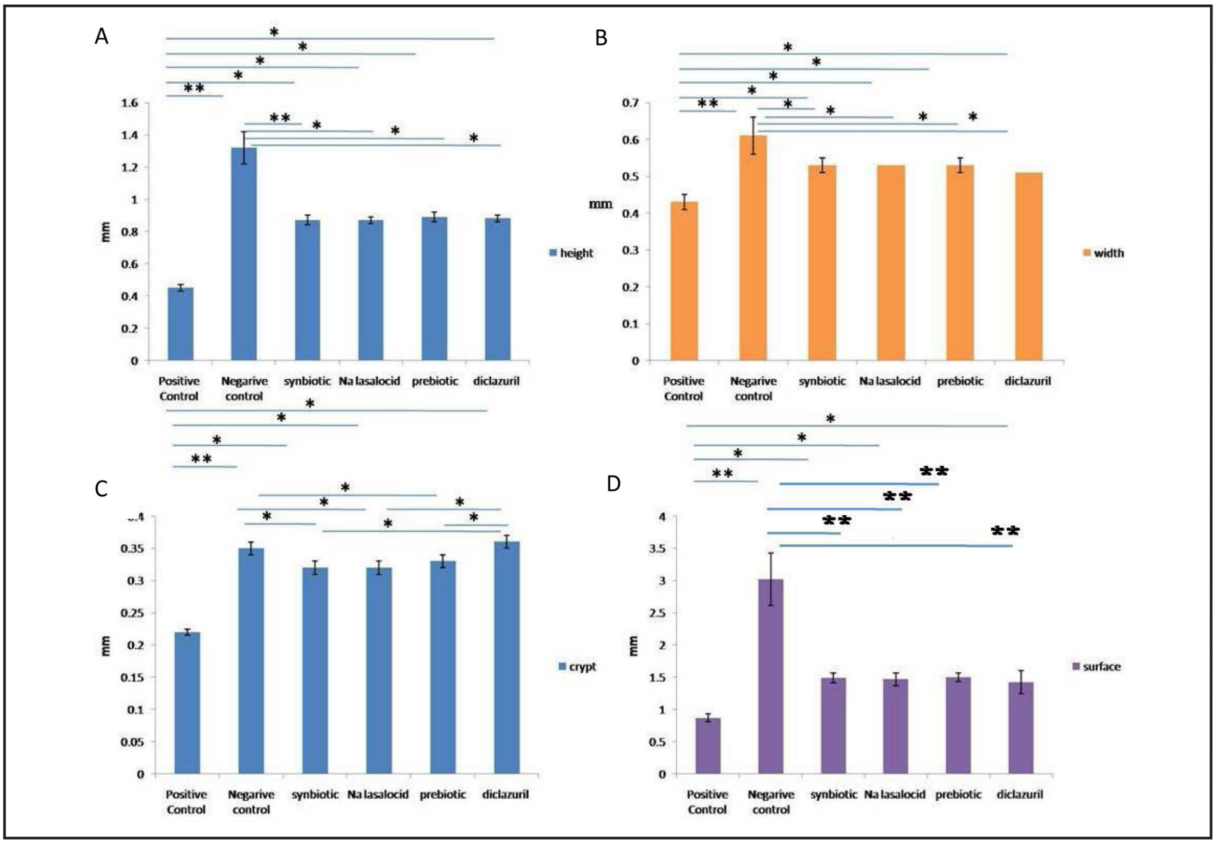

Figure 3. *Jeuhenum morphometric variables including height, width, and surface area of villus as well as depth of crypts on 28 day of age in various groups ** indicating P<0.001, * indicating P<0.01.*

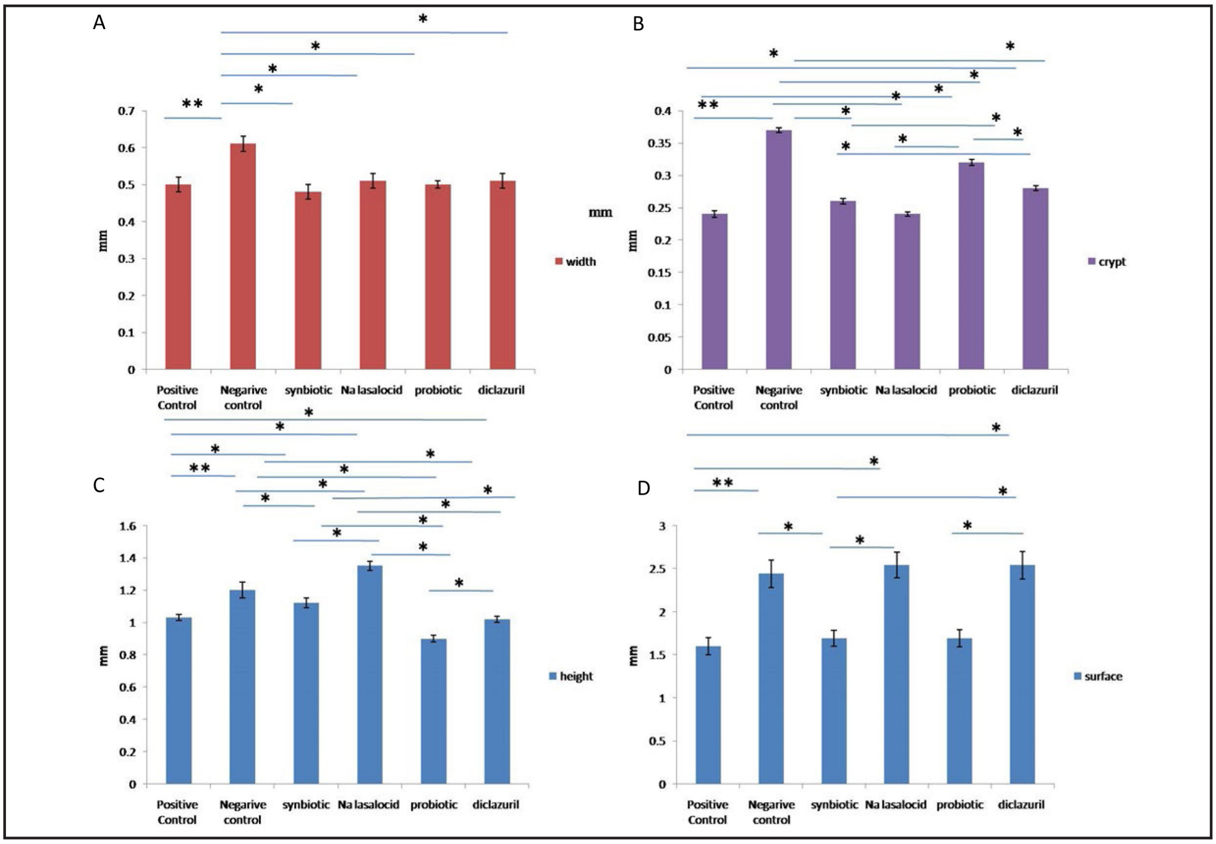

Figure 4. *Jeuhenum morphometric variables including height, width, and surface area of villus as well as depth of crypts on 49 day of age in various groups ** indicating P<0.001, * indicating P<0.01.*

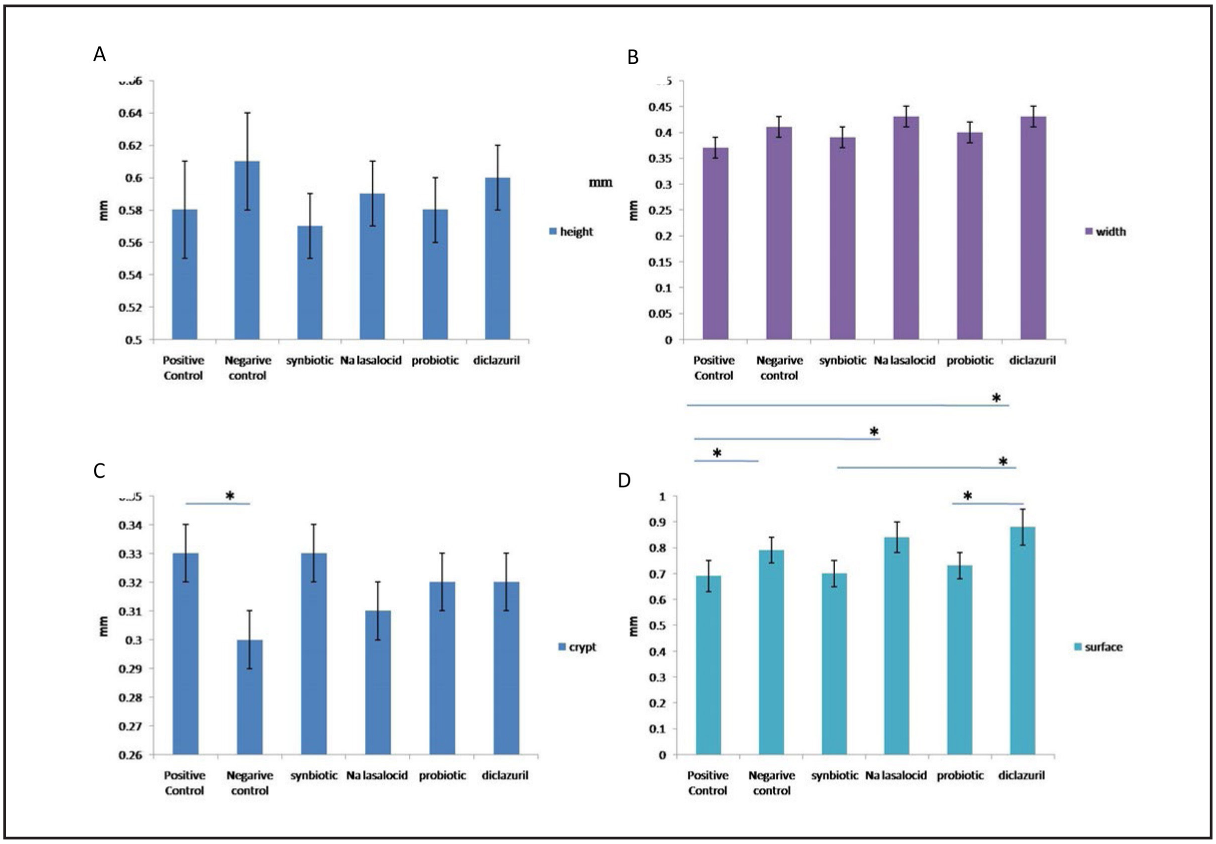

Figure 5. *Ileal morphometric variables including height, width, and surface area of villus as well as depth of crypts on 28 day of age in various groups ** indicating P<0.001, * indicating P<0.01.*

The lasalocid and probiotic groups showed significantly higher and lower height villi than those of other groups, respectively (P<0.05) (Fig .4c). A significant decrease was found in the synbiotic and diclazuril groups compared to the negative control respected to villi height.

The surface area **was lower rather than decreased** in the positive control, probiotic and synbiotic groups compared to other group.

In addition, the negative control, lasalocid, and diclazuril groups had significantly the most surface area amongst all groups (P<0.05)(Fig. 4d).

Ileum morphology findings on day 28 of age

There was no significant difference between all group in respected to ileal villi height and width (Fig. 5a, 5b).

A significant increase in the depth of crypt was only found in the positive group compared to the negative group.

The surface area significantly decreased in the positive control compared to the negative control, lasalocid, and diclazuril groups, as well as the synbiotic group compared to the diclazuriland probiotic groups (P<0.05)(Fig. 5c, 5d).

Ileum morphology findings on day 49 of age

There was a significant difference between all group in respected to ileal villi height and width in the positive group compared to all groups. The probiotic and diclazuril groups showed significantly lower height villi than those of other treatment and the negative control groups.

The lasalocid group had significant villi width compared to other groups.

The crypt depth of villi in the probiotic and diclazuril was significantly increased compared to all groups.

In contrast, the crypt depth of villi in the lasalocid and synbiotic was significantly decreased compared to all groups.

There was no significance difference in term of surface area amongst all groups (P<0.05) (Fig. 6a, 6b, 6c, 6d).

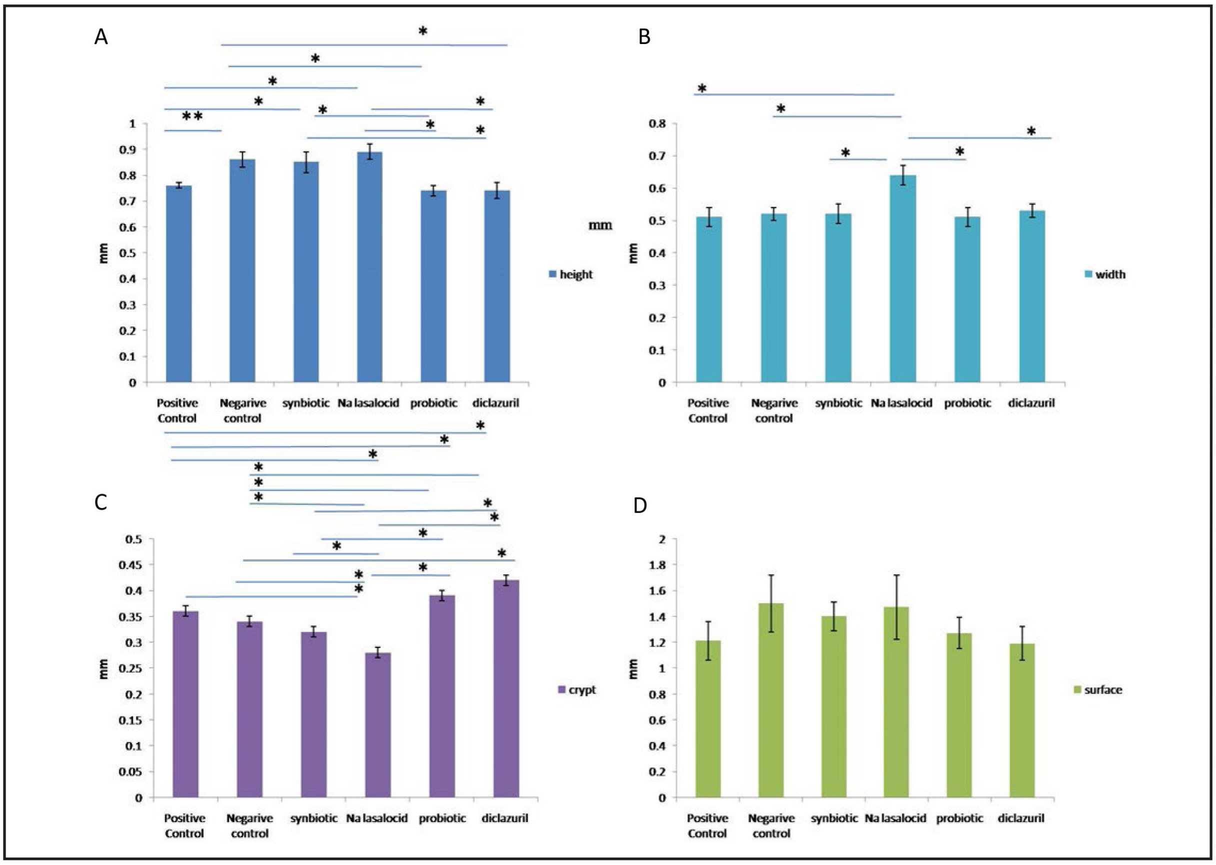

Figure 6. *Ileal morphometric variables including height, width, and surface area of villus as well as depth of crypts on 49 day of age in various groups ** indicating P<0.001, * indicating P<0.01.*

Discussion

Avian coccidiosis is a costly intestinal disease leading to inefficient nutrient absorption induced by considerable tissue damage, predisposition to secondary infections, and increasing of mortality rates (Calik *et al.* 2019). The primary objective of this study was to evaluate whether anti-coccidial drugs such as diclazuril and Na lasalocid show different therapeutic effects on intestinal morphology as well as growth performance compared to probiotic and synbiotic in broiler chicken with coccidial infection on days 28 and 49 of age.

Recent studies have shown that the probiotic supplementation can decrease the adverse effects of coccidial infection and improve the growth performance (El-Sawah *et al.* 2020). Co-administration of coccidiosis vaccine and probiotics has been shown to increase the protection against an *Eimeria* infection in birds (Ritzi *et al.* 2016). Probiotics have been confirmed to control the development of coccidiosis and reduce oocyst shedding (Travers *et al.* 2011).

The diclazuril causes transmembrane potential disruption of merozoites mitochondria ultrastructurally (Zhou *et al.* 2010). It has been shown that diclazuril downregulates mRNA expression level of the serine/threonine protein phosphatase type 5 (PP5) in Eimerian parasite (Zhou *et al.* 2013). Synbiotic has been demonstrated to improve performance, biochemical and haematological responses, and relative organ weights of laying hens fed diets supplemented with synbiotic (Tang *et al.* 2017)

Maintaining bird health, regarding diseases or agents acting on the gastrointestinal tract, is crucial in broiler production, since this is the entry route of nutrients for bird development. The small intestine is responsible for the digestion and absorption of nutrients from food, and the duodenal segment mainly for absorption (Assis *et al.* 2014). Broilers exhibiting shortening of villi have impaired nutrient absorption (Assis *et al.* 2014). In birds, cell divisions in the intestine, occur not only in crypts but also along villi, unlike in mammals.

The ideal intestinal morphometry in birds consists of long villi and shallow crypts. That is, length of villi is related to the digestive capacity and intestinal absorptive area (Uni *et al.* 2000). However, factors such as dietary supplements, drugs or pathogens can cause changes in the intestinal morphology.

Currently, it is common to use anicoccidials as prophylactic or treatment throughout the entire growing period in chicken to achieve total continual prevention of occurrence or suppression of coccidiosis. In the present study, we used lasalocid, diclazuril, probiotic and synbiotic in the diets of infected chickens and found that these drugs could not completely prevent the villus atrophy of small intestine induced by coccidiosis, although lasalocid and diclazuril partially improved this atrophy in the jejunum.

Previous studies have explained that anti-coccidial drugs have a narrow margin of safety and some of them are toxic. Even, their adverse effects on the bird performance (*i.e.* body weight and feed conversion rate) have been reported (Hassanpour *et al.* 2010). These adverse effects were different for each drug and depends on the dosage administered. However, as soon as *Eimeria* spp. infections build up in the flocks, the possible growth depressing effect of the drug will be neutralized by an effective control of the infection.

The compensatory growth observed after withdrawal of the drug from the feed, could also be explained by the growth depression effect of the drug used in their study (Tipu *et al.* 2002). Brake *et al.* (2001) also reported adverse effects of semduramicin, on broiler breeders. They found that semduramicin causes dose-related decrease in egg production, percentage shell, fertile hatchability and increase in early embryonic mortality. Hassanpour *et al.* (2010) reported that diclazuril, semduramicin, salinomycin and maduramycin, when used as prophylactic drugs against chicken coccidiosis, intestinal morphometric parameters (especially villus dimensions and absorptive surface) are severely diminished. Bahadoran *et al.* (2014) also found that Clopidol and Amprolium + Ethopabate decreased villous length, width and surface area, in the duodenum, jejunum and ileum. These findings indicated impaired nutrient absorption in the intestine and reduced enteric function due to anticoccidial drugs. They also reported a reduction of lamina propria thickness in the duodenum and jejunum by anticoccidials, which showed a deminished Lieberkühn's glands in these segments of intestine. Thus, it was predictable that these drugs may influence intestinal secretion in chickens.

In the present study, the decreased depth of duodenal lieberkuhn crypts by diclazuril and lasalocid as well as the decreased duodenal villus surface area by the probiotic and diclazuril on day 28 of age was reported. Probiotic and synbiotic also led to the atrophy of duodenal villi height and width, respectively. The negative effects of these drugs were less in the jejunum.

In conclusion, the economic losses resulted from coccidial infection in the poultry industry are caused by the decreased performance of broiler chicken induced by morphological changes in the any of the three segments of small intestine, including duodenum, jejunum, and ileum, specially jejunum. The morphological changes in the jejunum and ileum are started sooner/severe and later/mild than those other segments, respectively.

The anticoccidial drugs, synbiotic and probiotic can partially mitigate the morphological changes in any three segments of small intestine in broiler chicken with coccidiosis.

Since the jejunum morphological changes are starting sooner than other parts, and surface area of jejunal villi is important for nutrition absorbance, as well as growth performance, lasolacid was better than others treatment in this regard. Further research is needed to evaluate the effect of combination treatments.

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