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Effect of colistin and tylosin used as feed additives on the performance, diarrhea incidence, and immune response of nursery pigs

Efeito do uso da colistina e da tilosina como aditivos alimentares sobre o desempenho, incidência de diarreia e resposta imune de leitões na fase de creche

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Abstract

For the last several decades, antimicrobial compounds have been used as feed additives to promote piglet growth at weaning, through the prevention of subclinical and clinical disease. However, few studies have assessed the influence of these antibiotics on the immune response of nursery pigs, as well as the relation between performance, health, and immunity of animals that receive feed additives. Therefore, the present study aimed to evaluate the effects of colistin and tylosin when used as feed additives on the performance, incidence of diarrhea, and immune response of nursery pigs. In this study, 72 weaned pigs (average age, 28 days) were allotted into one of three treatment groups: a control group (feed with no antibiotics), tylosin group (feed containing 22 ppm tylosin), and colistin group (feed containing 20 ppm colistin). Weekly, during a five week period, the average daily feed intake, average daily gain, and feed conversion ratio of the pigs were evaluated. Stools were scored daily, in accordance with a fecal texture scale. Blood samples were collected on the day of housing (d0) and on d7, d21, d28, and d35 for immune cell phenotyping. The results of this study showed that piglets in both the colistin and tylosin groups exhibited a significantly higher average daily feed intake, resulting in a higher body weight at the end of the experimental period (d35) when compared with piglets from the control group. Colistin and tylosin also significantly reduced the incidence of diarrhea. Colistin and tylosin modulated the piglets' immune responses, particularly on d28, by changing the percentage of circulating B lymphocytes, CD4⁺CD8⁺ T cells, and the CD4:CD8 ratio.

Key words: Antibiotics. Cell-mediated immunity. Growth promoters. Pigs. Weaning.

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Resumo

Nas últimas décadas, os compostos antimicrobianos têm sido utilizados como aditivos alimentares para promover o crescimento de leitões ao desmame, por meio da prevenção de doenças clínicas e subclínicas. No entanto, poucos estudos avaliaram a influência destes antibióticos sobre a resposta imune de leitões na fase de creche, bem como a relação entre o desempenho, a saúde e a imunidade dos animais com o uso destes aditivos. Sendo assim, o presente estudo teve como objetivo avaliar o efeito da colistina e da tilosina como aditivos na alimentação sobre o desempenho, incidência de diarreia e resposta imune de leitões na fase de creche. Para isso, foram utilizados 72 suínos recém-desmamados, com 28 dias de idade em média, divididos em três tratamentos: grupo controle (ração sem antibiótico), grupo tilosina (ração com 22 ppm de tilosina), grupo colistina (ração com 20 ppm de colistina). Semanalmente, durante cinco semanas, foi analisado o consumo de ração diário, o ganho de peso diário e a conversão alimentar dos leitões. As fezes foram avaliadas diariamente, com a atribuição de escores relacionados à sua consistência. Amostras de sangue foram coletadas no momento do alojamento (d0) e aos d7, d21, d28 e d35, sendo submetidas à técnica de citometria de fluxo. Os resultados demonstraram que tanto o grupo colistina como o grupo tilosina promoveram aumento significativo no consumo de ração diário, o que se refletiu em maior peso ao final do estudo (d35), quando comparados ao grupo controle. A colistina e a tilosina também promoveram redução significativa na incidência de diarreia. A colistina e a tilosina modularam a atividade imune, principalmente aos 28 dias, alterando a porcentagem circulante de linfócitos B, células CD4+CD8+ e da razão entre CD4 e CD8.

Palavras-chave: Antibióticos. Desmame. Imunidade celular. Promotores de crescimento. Suínos.

Introduction

In industrial pig production, animals often experience stressful situations that may negatively affect their intestinal microbiota, allowing for the development of gastrointestinal infections. The time of weaning and the post-weaning period both demand a considerable effort by pig producers to reduce the stress experienced by piglets (GAGGIÀ et al., 2010). Those periods are characterized by a sudden – albeit transitional – reduction in feed intake, which negatively affects a piglet's growth potential, as well as increases their susceptibility to intestinal disorders and infections, resulting in diarrhea (MODESTO et al., 2009; HEO et al., 2013).

On most pig farms, piglets are weaned between 21 and 28 days of age (LALLÈS et al., 2007; MARCOLLA; RIBEIRO, 2015). This period corresponds with the transition from passive to active immunity, a time characterized by a dramatic reduction of blood IgG levels (BAUER et al., 2006), which increases an animal's susceptibility to infections. In addition, at the time of weaning, piglets experience several stressful situations, such

as a sudden change in the composition and physical form of their diets (nutritional stress), separation from the sow and the establishment of a new social hierarchy when different litters are mixed within nursery pens (social stress), as well as adapting to a new environment (environmental and handling stress) (MODESTO et al., 2009; PLUSKE, 2013; MARTINEZ et al., 2014). Both the compromised immune status and the stresses to which the piglets are exposed to favor the replication of pathogens in the intestines, which may result in diarrhea (YUAN et al., 2006; HEO et al., 2013; PLUSKE, 2013). Thus, it is common to include antimicrobials in the feed, used as performance enhancing additives, for piglets in the post-weaning phase (CROMWELL, 2002; HEO et al., 2013; PLUSKE, 2013).

Feed additives are defined as substances that are not nutrients that may be added to an animal's feed (PALERMO NETO, 2001). The main functions of feed additives are to increase productivity, to reduce mortality, and to prevent infections and feed spoilage (UTIYAMA et al., 2006). Antibiotics have been used as feed additives in livestock diets since 1949 (JUKES, 1972), particularly during

critical production phases, such as the weaning period in pigs. The efficiency of antibiotics in increasing growth rate, improving feed utilization, and reducing mortality from clinical disease is well documented (CROMWELL, 2002).

Colistin and tylosin are commonly used as feed additives in pig production. Colistin belongs to the polymyxin group of bactericidal antibiotics (SPINOSA, 2006a; MENDES; BURDMANN, 2009). It is produced by *Bacillus colistinus* (MENDES; BURDMANN, 2009) and acts against several Gram-negative microorganisms, including *Escherichia coli*, *Salmonella* spp., and *Pseudomonas* spp. (ZUANON et al., 1998). Polymyxins bind to phosphate radicals on the cell membranes of bacteria, which disrupts the cell membrane, resulting in a loss of the membrane's selectivity and allows for the leakage of small particles from the bacterial cytoplasm and eventually causing cell death. Tylosin is a bacteriostatic macrolide antibiotic isolated from *Streptomyces fradiae*. It inhibits microbial protein synthesis by interfering with the base of the translocation between A and P sites of the 50S unit of the bacterial ribosome, preventing tRNA translocation and inhibiting the enzyme peptidyl transferase, thereby preventing the elongation of the peptide chain (SPINOSA, 2006b).

The use of colistin and tylosin as antimicrobial feed additives is authorized in Brazil (MAPA, 2014). While some authors observed better performance in pigs fed colistin (LOVATTO et al., 2005; COSTA et al., 2007) and tylosin (YAN et al., 2011; GAVIOLI, 2013) as in-feed additives, others did not detect any performance differences in pigs fed either colistin (GOMES et al., 1981) or tylosin (VAN LUNEN, 2003), demonstrating the need of further studies on the efficacy of these antimicrobials. In addition, no literature reports were found that studied the effect of colistin and tylosin on cell-mediated immunity of nursery pigs, or the relation between performance, health, and immunity of animals that received

these feed additives. Therefore, this study aimed at evaluating the effects of colistin and tylosin when used as feed additives on the performance, incidence of diarrhea, and immune response of nursery pigs.

Materials and Methods

Animals and facilities

Seventy-two recently weaned piglets, with an average age of 28 days, were allotted into one of three treatment groups with eight replicates of three animals each, according to a randomized block experimental design. The duration of the experimental period was five weeks, starting at the time of housing.

Pigs were housed in suspended nursery pens (1.2 × 1.6 m), with fully-slatted plastic flooring, and were offered feed and water *ad libitum* during the entire experimental period. Pigs were handled according to the practices recommended by the Canadian Council on Animal Care (CCAC, 1993). The experimental procedures were approved by the Ethics Committee on the Use of Animals of Pontifical University Catholic of Parana (PUCPR), under project number 792.

Diets

The feeding period was divided into a pre-starter phase (first two weeks of the experiment) and a starter phase (last three weeks of the experiment). A basal diet was formulated for each feeding phase (Table 1) to meet or exceed the nutritional requirements of nursery pigs as determined by the National Research Council (NRC, 2012). Calculated nutritional values from pre-starter and starter diets can be observed in table 2. The three experimental diets consisted of: the basal diet without antibiotics (control group), the basal diet with the addition of 20 ppm of colistin (colistin group), and the basal diet with the addition of 22 ppm of tylosin (tylosin group).

Performance parameters

Piglets were weighed at the time of housing and were then evenly distributed into the experimental

treatment groups according to their body weight. Average daily feed intake (ADFI), average daily gain (ADG) and feed conversion ratio (FCR) were recorded weekly.

Table 1. Composition of the pre-starter and starter diets used in the post-weaning phases.

Ingredient	Pre Starter (g/kg)	Starter (g/kg)
Corn	435.9	513.0
Soybean meal	260.0	250.0
Premix ¹	276.8	202.7
Soybean oil	15.0	17.0
Limestone	9.0	9.0
Dicalcium phosphate	-	3.0
L-Lysine	2.0	3.8
DL-Methionine	0.4	0.5
Threonine	0.9	1.0
TOTAL	1000	1000

¹Guaranteed levels per kg of product: folic acid (min) 2.40 mg; pantothenic acid (min) 88.00 mg; biotin (min) 0.40 mg; calcium 16.44 g; copper (min) 800 mg; ether extract (min) 75.60 g; iron (min) 800 mg; crude fiber 5.70 g; phosphorus (min) 14.93 g; iodine (min) 7.2 mg; lysine (min) 16.95 g; manganese (min) 220 mg; mineral matter (max) 119.35 g; methionine (min) 700 mg; niacin (min) 140.00 mg; crude protein (min) 155.72 g; selenium (min) 1.20 mg; sodium (min) 12.22 g; threonine (min) 10.36 g; tryptophan (min) 2.930 mg; wetness (max) 62.30 g; vitamin A (min) 50,000.00 IU; vitamin B1 (min) 12 mg; vitamin B12 (min) 100 µg; vitamin B2 (min) 20 mg; vitamin B6 (min) 12 mg; vitamin D3 (min) 10,000.00 IU; vitamin E (min) 160.00 IU; vitamin K3 (min) 12.00 mg; and zinc (min) 500.00 mg.

Table 2. Calculated nutritional values of the pre-starter and starter diets used in the post-weaning phases.

Ingredient	Pre-starter	Starter
Crude protein (%)	19.54	18.53
Crude fat (%)	2.29	2.33
Lactose (%)	6.03	4.42
Crude fiber (%)	0.87	0.82
Calcium (%)	0.68	0.63
Phosphorus (%)	0.46	0.41
Sodium (%)	0.34	0.25
Chlorine (%)	1.49	1.54
Lysine (%)	0.44	0.4
Methionine+Cysteine (%)	0.74	0.69
Threonine (%)	0.25	0.23
Choline (%)	0.89	0.83
Metabolizable Energy (kcal/Kg)	3341.5	3335.06

Diarrhea incidence

Pigs were inspected daily in the morning to monitor the fecal score of all pens, and the fecal texture was scored according to a 0-3 scale where 0 = normal stools, 1 = soft stools, 2 = slurry stools, and 3 = watery stools. Scores 0 and 1 were considered normal stools, and scores 2 and 3 were considered diarrhea. The evaluation of the incidence of diarrhea was carried out by comparing the number of stools with scores of 2 and 3 between different treatment groups.

Immune cell profile

In order to evaluate the possible effects of colistin and tylosin on the cellular immune response of piglets, blood immune cells were phenotyped. Blood samples were collected from one individual per replicate at the time of housing (d0), and at d7, d21, d28, and d35 of the experiment.

Specific monoclonal antibodies including anti-CD4 (conjugated with fluorescein isothiocyanate, FITC), anti-CD8 (conjugated with phycoerythrin, PE), anti-monocyte+macrophage Kul-1 (conjugated with fluorescein isothiocyanate, FITC), and anti-B lymphocytes (conjugated with phycoerythrin, PE) were used. All antibodies were acquired from Southern Biotech (Birmingham, AL, USA), and were produced from mice.

Blood samples were processed according to Fernandes Filho et al. (2013) and analyzed in a flow cytometer (FACSCalibur, Becton Dickson, Franklin Lakes, NJ, USA). FITC fluorescence was detected in the FL1 channel (nm 530/30) and PE fluorescence in the FL2 channel (nm 585/42). At least 10,000

events were acquired based on the selection of the lymphocyte region on the cell size and complexity graph (FSC x SSC). Flow cytometry data were analyzed using the CellQuest Pro software (Becton Dickson, Franklin Lakes, NJ, USA).

Statistical analysis

Data were submitted for analysis of variance and means were compared by the LSD test, using the Statistix 8 statistical package (Analytical Software, Tallahassee, FL, USA).

Differences in fecal scores were evaluated with the Kruskal-Wallis test, followed by the Dunn's multiple comparison test.

Immune cell percentages were submitted for analysis of variance and means were compared by the test of Bonferroni. Graphs and statistical analyses were generated by the software GraphPad Prism (GraphPad Software, La Jolla, CA USA).

All statistical analyses were carried out at $p < 0.05$.

Results and Discussion

Average performance results of the control, tylosin, and colistin groups obtained for the first two experimental weeks (d14) and for the total experimental period (d35) are shown in Table 3 and 4 respectively.

Average daily feed intake (ADFI), average daily gain (ADG) and feed conversion ratio (FCR) were not influenced by the treatments during the first two experimental weeks. On d14, piglets in the tylosin and colistin groups were significantly heavier than those in the control group.

Table 3. Average initial body weight (AW1), average daily feed intake (ADFI), average daily gain (ADG), feed conversion ratio (FCR), and body weight on day 14 post weaning (W14) of nursery pigs fed a basal diet without antibiotics (control group) or with 20 ppm of colistin (colistin group) or with 22 ppm of tylosin (tylosin group).

Treatments	AW1 (kg)	ADFI (kg)	ADG (kg)	FCR	W14 (kg)
Control group	5.92	0.447	0.302	1.50	10.19 ^b
Tylosin group	5.92	0.507	0.361	1.40	10.98 ^a
Colistin group	5.95	0.504	0.362	1.41	11.02 ^a
P value	0.344	0.115	0.053	0.114	0.041
Coefficient of variation	0.86	12.39	14.96	7.07	6.10

Different superscripts in the same column indicate significant differences by the LSD test ($p \leq 0.05$).

Table 4. Average daily feed intake (ADFI), average daily gain (ADG), feed conversion ratio (FCR), and body weight on day 35 post weaning (W35) of nursery pigs fed a basal diet without antibiotics (control group) or with 20 ppm of colistin (colistin group) or with 22 ppm of tylosin (tylosin group).

Treatments	ADFI (kg)	ADG (kg)	FCR	W35 (kg)
Control group	0.794 ^b	0.474	1.67	22.56 ^b
Tylosin group	0.880 ^a	0.525	1.67	24.30 ^a
Colistin group	0.885 ^a	0.520	1.70	24.24 ^a
P value	0.035	0.069	0.635	0.050
Coefficient of variation	8.25	8.72	4.09	6.11

Different superscripts in the same column indicate significant differences by the LSD test ($p \leq 0.05$).

The results presented in Table 4 show that the pigs in the tylosin and colistin groups averaged a significantly higher ADFI during the entire experimental period when compared with those in the control group. This feed intake difference resulted in a different body weight at the end of the experiment (d35), as the pigs in the tylosin and colistin groups were significantly heavier than the control animals. In addition, pigs in the tylosin and colistin groups also exhibited a higher, albeit not statistically significant ($P = 0.069$), average daily gain (ADG) during the total experimental period (d35) compared with those in the control group. The feed conversion ratio was not significantly different among treatments.

Tylosin is able to alter the intestinal microbiota, and its use in piglets is related to an improvement in animal performance, especially related to feed intake as observed in the present study, and feed conversion ratio (BOSI et al., 2011; YAN et al., 2011; KIM et al.,

2012; GAVIOLI, 2013). One mechanism that may explain this improved performance is an increase in the relative mass of lactobacilli in the intestinal tract when compared to enterogenic bacteria, owing to a significant decrease of the latter (COLLIER et al., 2003; BOSI et al., 2011). Therefore, because of the prevalence of lactobacilli within the small intestine, where the majority of nutrient absorption occurs, the alteration of these bacteria is a potentially important mechanism involved in antibiotic-influenced animal growth (COLLIER et al., 2003). Macrolides are known to be minimally effective against Gram-negative bacteria in the intestine because of the outer membrane permeability barrier of Gram-negative bacteria. The outer membrane cannot be easily penetrated by macrolides due to the increased molecular size of these antibiotics in relation to the size of the bacterial porin channels (NORCIA et al., 1999). Nevertheless, some enterobacteria strains have an incomplete

lipopolysaccharide structure, permitting increased penetration by macrolides (NORCIA et al., 1999). There is also evidence that this class of antibiotics can directly interact with the immune response after induction by lipopolysaccharides; however, in that instance, macrophages and monocytes responded by decreasing the gene expression of various inflammatory cytokines (CAO et al., 2006). Thus, the positive action of tylosin on piglet growth and feed intake observed in the present study can result from the decreased costs of immune activation determined by the action on intestinal microflora or from the enhanced uptake and use of nutrients.

Gavioli (2013) carried out a study comparing the performance of growing pigs that were fed diets containing colistin (10 ppm), tylosin (40 ppm), or a symbiotic product, and found a higher average daily feed intake in pigs fed tylosin when compared to other treatments, but there were no significant differences in other evaluated performance parameters. Those authors report that the higher average daily feed intake in pigs fed tylosin compared to pigs fed colistin may be due to sensory characteristics of these antibiotics, as colistin incorporates a bitter taste to the feed, leading to a reduction in dietary intake. However, this reduction was not observed in our study. On the other hand, other authors did not observe any improvements in the performance of nursery pigs with the inclusion of tylosin in the feed (SILVA et al., 2007; HOLMAN; CHÉNIER, 2013).

The results obtained with colistin in the present study are consistent with the findings of Lovatto et al. (2005), who obtained a higher average daily feed intake and average daily gain when colistin was added to the diet of nursery pigs. Sbardella (2014) observed that the use of colistin as a feed additive (at 40 ppm) improved the final body weight, weight gain, and feed conversion ratio of nursery pigs. However, these authors did not find any effect of colistin on feed intake, in contrast to the findings of the present study.

How antibiotics, used as feed additives, increase performance is not clear, but possible mechanisms

may include: a reduction in total bacterial load; suppression of pathogens; inhibition of endemic subclinical infection, thus reducing the metabolic costs of the (innate) immune system; reduction of growth-depressing metabolites (such as ammonia and bile degradation products) produced by microbes; reduction of microbial use of nutrients; enhancing the uptake and use of nutrients, because the intestinal wall in animals fed with feed additives is thinner; and direct modulation of the immune system (DIBNER; BUTTIN, 2002; HARDY, 2002; BUTAYE et al., 2003; DIBNER; RICHARDS, 2005; NIEWOLD, 2007). According to Niewold (2007), antibiotics most likely work as growth promoters by inhibiting the production and excretion of catabolic mediators by intestinal inflammatory cells. Concomitant or subsequent changes in microflora are most likely the consequence of an altered condition of the intestinal wall. It is suggested that the use of antibiotics decreases the level of inflammation. Intestinal inflammation usually leads to an accumulation of inflammatory cells in the intestinal mucosa, leading to a thicker intestinal wall. The thinner intestinal wall observed when using antibiotics as feed additives is consistent with a reduction in inflammation because of the reduced influx and accumulation of inflammatory cells (LARSSON et al., 2006). Moreover, inflammation and cytokine release promotes catabolism of muscle tissue and loss of appetite (GRUYS et al., 2006), which additionally affects an animal's performance.

Torrallardona et al. (2003) compared spray-dried plasma and colistin (300 ppm) as feed additives for weaned piglets and showed that both products improved performance. In particular, colistin improved ADG, ADFI, and FCR, which is in agreement with the results of the present study. Those authors also observed that colistin helped to maintain the integrity of the intestinal mucosa, as suggested by the lower small intestinal weight and longer intestinal villi of the pigs fed colistin. In addition, colistin reduced the number of enterococci in the cecum and of *Escherichia coli* both in the ileum and in the cecum.

The incidence of diarrhea was lower in the tylosin and colistin groups when compared with the control group (Table 5), and can be observed by the ranked mean of scores 2 and 3, which were considered diarrhea. Post-weaning diarrhea syndrome is a multifactorial disease that affects piglets after weaning. It may cause considerable economic losses, as mortality may reach up to 10% of pigs, in addition to increasing the number of culls, delaying growth, and increasing medication costs. The disease is also known as post-weaning colibacillosis, because the main causative agents are enterotoxigenic strains of

Escherichia coli (THOMSSON et al., 2008; LIMA et al., 2009). Other pathogens, such as *Lawsonia intracellularis*, *Salmonella typhimurium*, and *S. choleraesuis* may also be present and cause enteritis, thereby making the diagnosis and treatment of this condition difficult (MORÉS; MORENO, 2007). It has been suggested that the high susceptibility of early-weaned piglets to enteric disorders is due to disruption in the establishment of a stable intestinal microbiota, thereby allowing pathogenic bacteria to flourish and cause disease (KONSTANTINOV et al., 2004, 2006).

Table 5. Fecal score of nursery pigs fed a basal diet without antibiotics (control group) or with 20 ppm of colistin (colistin group) or with 22 ppm of tylosin (tylosin group) obtained by daily visual inspection of the pens during the 35 days of experiment. Stools were scored daily according to a fecal texture scale, where 0 = normal stools, 1 = soft stools, 2 = slurry stools, or 3 = watery stools.

Fecal score	Treatments		
	Control group	Tylosin group	Colistin group
0	978	937	1090
1	1081	1086	1088
(0+1) ^a	(2059)	(2023)	(2178)
2	373	197	290
3	182	48	102
(2+3) ^b	(555)	(245)	(392)
Weighted Mean	0.9078	0.7160*	0.7680*
Rank Mean ^c (all scores)	3940.29	3574.75*	3642.97*
Rank Mean ^c (scores 2+3)	625.95	547.27*	585.58*

^a Scores 0 and 1 were considered as normal, ^b Scores 2 and 3 were considered as diarrhea, ^c Means obtained by the Kruskal-Wallis test. * Indicates significant difference when compared with the control group (Dunn's multiple comparison test).

The maintenance of the integrity of the intestinal mucosa and the reduced *Escherichia coli* counts in the ileum and the cecum of weaning pigs with the use of colistin, proposed by Torrallardona et al. (2003), may explain the results concerning the incidence of diarrhea in the present experiment, in that diarrhea was significantly lower in the colistin group as compared with the control group (Table 5). Sbardella (2014) also found a lower incidence of diarrhea in nursery pigs fed colistin compared with the control animals. On the other hand, Luna et al. (2015) did not observe any influence of colistin dietary supplementation on the incidence of diarrhea in nursery piglets.

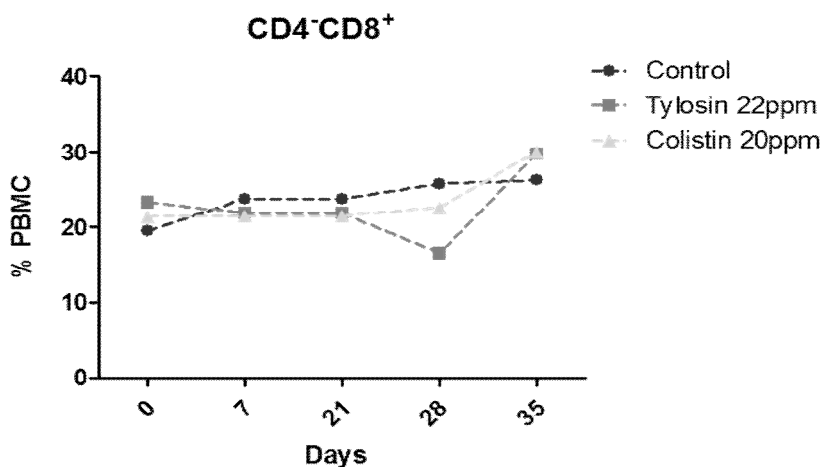
The reduction in the diarrhea incidence observed in the tylosin group (Table 5) may be due to the ability of this antimicrobial to alter the intestinal microflora, increasing the number of bacteria such as lactobacilli, as already mentioned (COLLIER et al., 2003; BOSI et al., 2011). Lactobacilli are the predominant lactic acid bacteria found in the pig intestine and constitute a major proportion of intestinal microbiota. As such, they are of particular importance to the maintenance of gut health. The presence and activity of lactobacilli have a stimulatory effect on both gut immunity and maturation, enhancing immune protection, and reducing gastrointestinal inflammatory responses

(KIMURA et al., 1997; BLUM; SCHIFFRIN, 2003). They also display antimicrobial activities that are involved in host epithelial immunity, such as the reduction of colonic pH (through the production of lactic acid), protection against mucosal pathogen invasion, and production of bacteriocins (VARCOE et al., 2003; PUTAALA et al., 2010). Kim et al. (2012) suggested that the development of the 'mature' gut microbiota in swine is accelerated by the addition of tylosin, although the gut microbiota of untreated pigs eventually reaches this state as well. However, Bosi et al. (2011) did not observe

any difference from tylosin dietary supplementation on the fecal score of nursery piglets.

Another possible reason for the variations in diarrhea incidence are changes in the immune profile of the piglets. The use of the additives had a marked effect on immune cell dynamics in peripheral blood. The results of the immune cell profiles are shown in the graphs below. No significant differences were observed in the percentages of circulating CD4⁺CD8⁺ T cells (Figure 1), circulating CD4⁺CD8⁺ cells (Figure 2), or macrophages (Figure 3).

Figure 1. Percentage of circulating CD4⁺CD8⁺ T cells in relation to peripheral blood mononuclear cells (PBMC). Nursery pigs were fed a basal diet without antibiotics (control) or with 20 ppm of colistin or 22 ppm of tylosin. Blood was collected for PBMC immune cell typing at the time of weaning (day 0) and 7, 21, 28, and 35 days later. The immunophenotype of PBMC was determined by flow cytometry.



Statistical differences were observed only on d28 for the populations of B cells (Figure 4), CD4⁺CD8⁺ T cells (Figure 5) and for the CD4:CD8 ratio (Figure 6).

On d28, piglets from the colistin group exhibited lower circulating B-cell percentages compared to those from other groups (Figure 4). Bouskra et al. (2008) showed that colistin reduced the proliferation of individual lymphoid follicles within the intestine of mice. These authors found that the peptidoglycans of Gram-negative bacteria, which are the target of colistin, are essential for the development

of intestinal lymphoid follicles. Therefore, it is possible that the effect of colistin on Gram-negative bacteria modulated the percentage of circulating immune cells, particularly B cells, in the present experiment. This is consistent with the findings that piglets fed colistin had lower leukocyte percentages (particularly of lymphocytes) compared with those fed plant extracts (SAVOINI et al., 2002). The modulation of the intestinal microbiota may reduce the activity of cells, essentially by controlling the exposure to microbial stimuli, as shown with the administration of the antibiotic polymixin (GORIS et al., 1986).

Figure 2. Percentage of circulating CD4⁺CD8⁻ T cells in relation to peripheral blood mononuclear cells (PBMC). Nursery pigs were fed a basal diet without antibiotics (control) or with 20 ppm of colistin or 22 ppm of tylosin. Blood was collected for PBMC immune cell typing at the time of weaning (day 0) and 7, 21, 28, and 35 days later. The immunophenotype of PBMC was determined by flow cytometry.

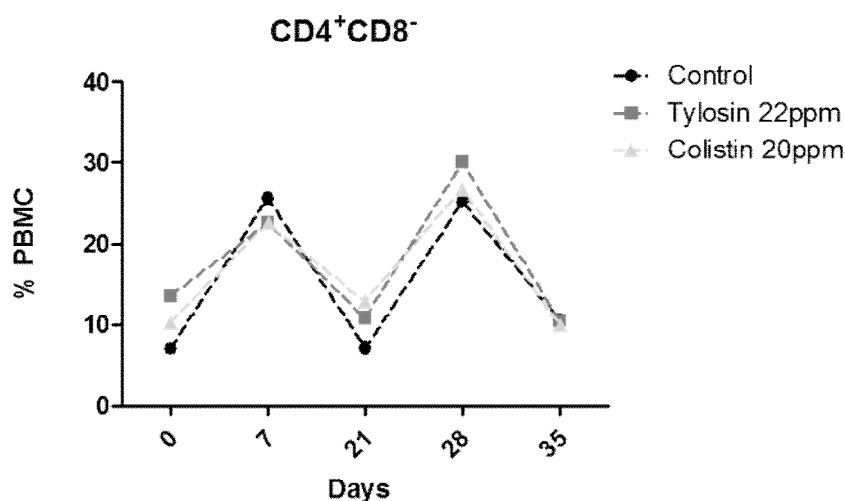


Figure 3. Percentage of circulating monocytes in relation to peripheral blood mononuclear cells (PBMC). Nursery pigs were fed a basal diet without antibiotics (control) or with 20 ppm of colistin or 22 ppm of tylosin. Blood was collected for PBMC immune cell typing at the time of weaning (day 0) and 7, 21, 28, and 35 days later. The immunophenotype of PBMC was determined by flow cytometry.

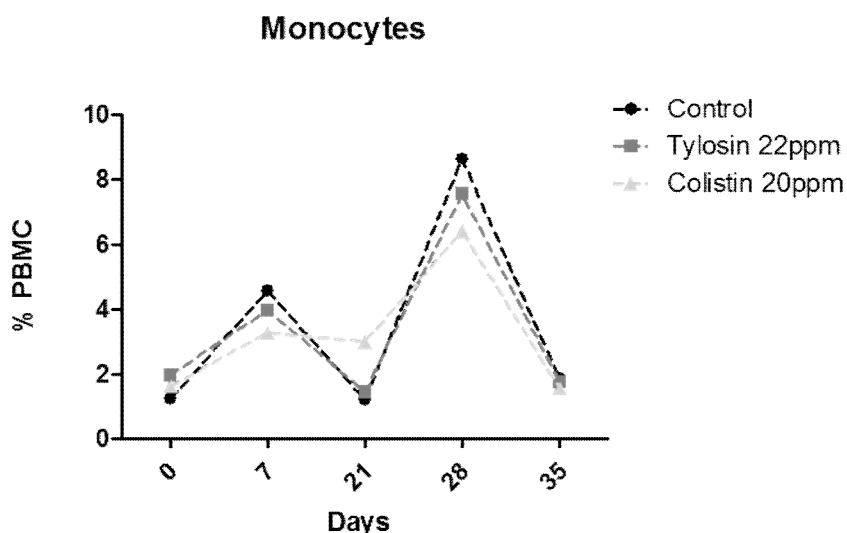
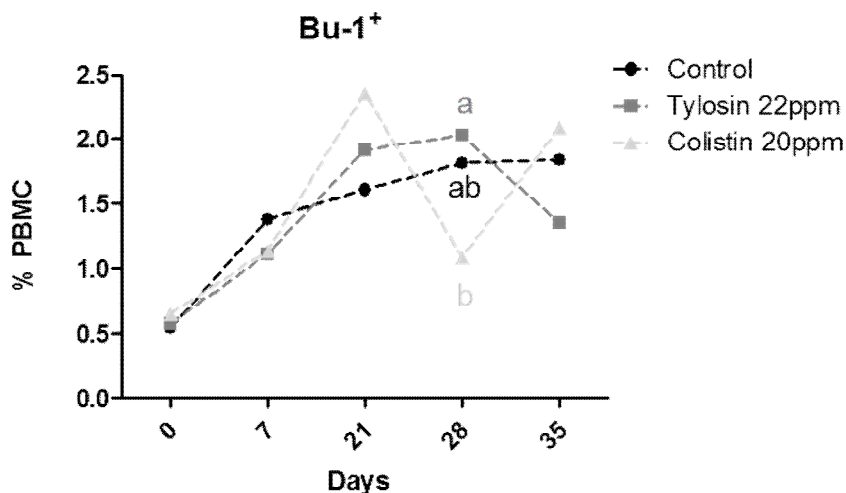


Figure 4. Percentage of circulating Bu-1⁺ cells (B-lymphocytes) in relation to peripheral blood mononuclear cells (PBMC). Nursery pigs were fed a basal diet without antibiotics (control) or with 20 ppm of colistin or 22 ppm of tylosin. Blood was collected for PBMC immune cell typing at the time of weaning (day 0) and 7, 21, 28, and 35 days later. The immunophenotype of PBMC was determined by flow cytometry. Statistical differences at a specific date are indicated by different letters with the same color as the corresponding group. Data were analyzed by two-way ANOVA ($P < 0.05$).



On d28, the control group showed a higher percentage of CD4+CD8⁺ T cells relative to the tylosin group, while the colistin group showed similar percentages to the former (Figure 5). Double-positive T cells (CD4+CD8⁺) are a specific immune characteristic of pigs. Many authors believe that they are activated memory cells (ZUCKERMANN; HUSMANN, 1996), capable of eliciting humoral immune responses (SAALMÜLLER et al., 2002). In a study with calves, the percentages of circulating CD4 and CD8 T cells were modulated with the use of tylosin (SZYMAŃSKA-CZERWIŃSKA et al., 2009). The double-positive population is rare in cattle, but the results of the present study with CD4+CD8⁺ T cells (Figure 5) may be consistent with the findings of the study of Szymańska-Czerwińska et al. (2009), which analyzed the individual CD4 and CD8 populations. In the present study, no differences in the percentages of the individual CD4 or CD8 T cells were observed (Figures 1 and 2); however, double-positive CD4+CD8⁺ T cells decreased when tylosin was used as a feed additive. This effect may be due to the effect of tylosin on macrophages, which in turn control

the subsequent immune response by producing cytokines (SZYMAŃSKA-CZERWIŃSKA et al., 2009). Although no difference was seen concerning the circulating numbers of monocytes, tylosin could have had an effect on cytokine production by these cells, or could even have altered local (intestinal, for instance) macrophage populations, which can control circulating lymphocytes. Tylosin is indeed capable of altering the proliferative capacity of immune cells, thus explaining some of the findings of the present study (BABA et al., 1998).

On d28, the CD4:CD8 ratio was higher in the tylosin group as compared to the colistin group. The lowest ratio was obtained in the control group (Figure 6). Tylosin promotes the production of pro-inflammatory cytokines, such as interleukin 1 (IL-1), interleukin 2 (IL-2), interferons (IFNs), and tumor necrosis factor alpha (TNF α). Szymańska-Czerwińska et al. (2009) showed that tylosin increased the percentage of CD4⁺ in calves, which may explain the higher CD4:CD8 ratio obtained in the present study in the piglets fed tylosin. In other species, it has been suggested that this ratio is indicative of immune competence (BRIDLE et al., 2006).

Figure 5. Percentage of circulating double-positive CD4⁺CD8⁺ T cells in relation to peripheral blood mononuclear cells (PBMC). Nursery pigs were fed a basal diet without antibiotics (control) or with 20 ppm of colistin or 22 ppm of tylosin. Blood was collected for PBMC immune cell typing at the time of weaning (day 0) and 7, 21, 28, and 35 days later. The immunophenotype of PBMC was determined by flow cytometry. Statistical differences at a specific date are indicated by different letters with the same color as the corresponding group. Data were analyzed by two-way ANOVA ($P < 0.05$).

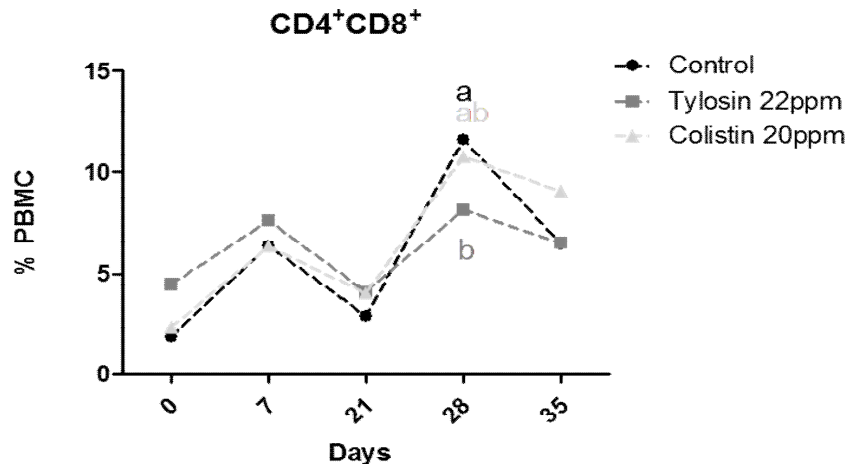
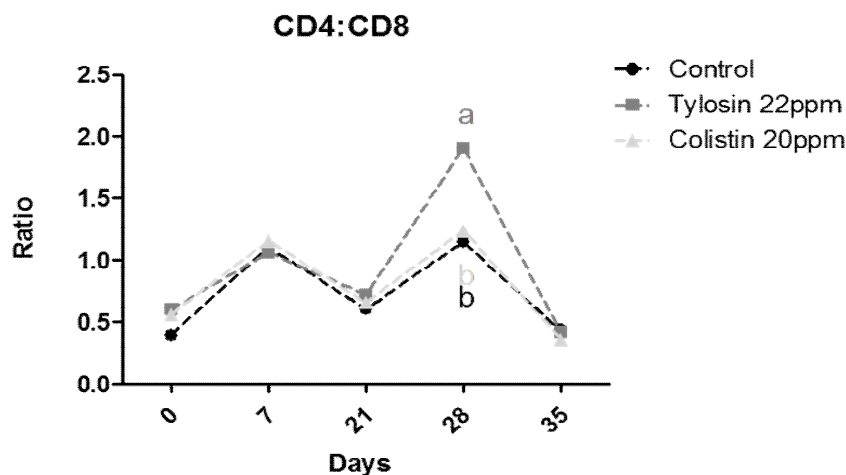


Figure 6. Percentage of circulating CD4 to CD8 T cell ratio. Nursery pigs were fed a basal diet without antibiotics (control) or with 20 ppm of colistin or with 22 ppm of tylosin. Blood was collected for PBMC immune cell typing at the time of weaning (day 0) and 7, 21, 28, and 35 days later. The immunophenotype of PBMC was determined by flow cytometry. Statistical differences at a specific date are indicated by different letters with the same color as the corresponding group. Data were analyzed by two-way ANOVA ($P < 0.05$).



The fluctuations in the immune cells were accompanied by an improvement in the health status of the treated groups. Two interpretations can be derived from this observation, either the immune changes are causes or consequences of the improved diarrhea status. The additives may have altered the immune stimulation by removing environmental pressure; conversely, the additives may have led

to an improved immune response, which itself suppressed the environmental pressure. The actual effect of these immune changes on the health status of the animals cannot be determined with the data presented here, and should be further clarified in the future with the use of experimental infections and long-term studies.

Conclusions

The inclusion of colistin and tylosin in the diet of nursery piglets as feed additives significantly increased average daily feed intake, resulting in a higher body weight at the end of the experimental period. Colistin and tylosin also promoted a significant reduction in the incidence of diarrhea, which probably contributed to the improved performance observed.

Colistin and tylosin modulated the piglet's immune response, particularly on d28, changing the percentage of circulating B lymphocytes, CD4⁺CD8⁺ T cells, and the CD4:CD8 ratio. It is important to observe that the immune changes were linked to a clinical finding (diarrhea), regardless of whether they were causative or consequential. The different mechanisms of action of both additives may account for the varying immune effects seen after using the additives.

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