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Chito-oligosaccharide as growth promoter replacement for weaned piglets: performance, morphometry, and immune system

Quitooligossacarídeo como substituto aos promotores de crescimento para leitões desmamados: desempenho, morfometria e imunidade

Eduardo Raele Oliveira[1]*; Caio Abércio da Silva[2]; Raúl Jorge Hernan Castro-Gómez[3]; Arturo Pardo Lozano[1]; David Fernandes Gavioli[1]; Juliana Frietzen[1]; Elisângela Olegário da Silva[1]; Aliny Ketilim Novais[1]; Giovani Frederico[4]; Marcino Pereira Júnior[1]

Abstract

The objective of this study was to evaluate the chito-oligosaccharide (COS) against two growth promoter antibiotics, colistin and lincomycin, with respect to growth performance, incidence of diarrhea, visceral characteristics, morphometry, and serum immunoglobulin levels (IgA, IgG, and IgM). A total of 96 Pen Ar Lan® piglets (48 barrows and 48 females), weaned at 17 days and with body weight (BW) 5.33 ± 0.37 kg, were subjected to the evaluation of growth performance and serum. Twenty-four animals, females, 35-day-old and with BW 6.86 ± 0.64 kg, were used for the assessment of histology and visceral organ weight. The three treatments were a basic diet formulation supplemented with COS (100 mg kg^{-1}), colistin (40 mg kg^{-1}), or lincomycin (4.4 mg kg^{-1}). The antibiotic treatments showed higher average daily gain (ADG) than COS treatment during the period of 49 to 63 days; whereas the feed conversion ratio (FCR) was higher and incidence of diarrhea was lower for the colistin treatment than for other treatments. The spleen weight and the small intestinal length were higher and duodenal pH was lower for COS than for antibiotics. Morphometry indicated greater villus height and higher ratio of villus height to crypt depth with colistin than with COS and a lower lesion score compared with other treatments. The serum IgA concentration was higher for COS in 35-day-old piglets. According to the results, COS was not efficient to replace colistin as a growth promoter for piglets weaned at 17 days; however, the results related to the immune system suggested that COS is a potentially promising product during weaning.
Key words: Colistin. Diarrhea. Histology. Immunoglobulins. Lincomycin.

Resumo

Realizou-se este trabalho com o objetivo de avaliar o quitooligossacarídeo (QOS) frente a dois antibióticos utilizados como promotores de crescimento, a colistina e lincomicina, quanto ao desempenho, frequência de diarreia, características viscerais, morfometria e imunoglobulinas séricas, IgA, IgG e IgM. Foram utilizados 96 leitões de genética Pen Ar Lan®, desmamados com idade média de 17 dias e peso de 5,33

[1] Discentes, Pós-graduação em Ciência Animal, Universidade Estadual de Londrina, UEL, Londrina, PR, Brasil. E-mail: eraele@outlook.com; setaarturo@hotmail.com; dgavioli@deheus.com; jufrietzen@uel.br; eli_olegario@homtail.com; alinyketilim@hotmail.com; marcino@deheus.com

[2] Prof. Dr., Programa de Pós-Graduação em Ciência Animal, UEL, Londrina, PR, Brasil. E-mail: casilva@uel.br

[3] Prof., Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, UEL, Londrina, PR, Brasil. E-mail: rcastrog@yahoo.com

[4] Discente, Curso de Medicina Veterinária, UEL, Londrina, PR, Brasil. E-mail: giovanifre@hotmail.com

* Author for correspondence

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± 0,369 kg para avaliação de desempenho e imunoglobulinas séricas. Para a avaliação histológica e peso dos órgãos, foram utilizados 24 animais com idade de 35 dias e peso de 6,86 ± 0,64 kg. Os três tratamentos consistiram em uma dieta basal acrescida de QOS (100 mg Kg^{-1}), dieta basal com colistina 40 mg Kg^{-1} e dieta basal com lincomicina 4,4 mg Kg^{-1}. Para o desempenho, os antibióticos apresentaram melhor ganho diário de peso (GPD) dos 49 aos 63 dias de idade em relação ao QOS e a conversão alimentar (CA) foi melhor para a colistina em comparação com os demais tratamentos, além disso apresentou menor incidência de diarreia aquosa quando comparada com os outros tratamentos. Para as vísceras, observou-se aumento do peso relativo do baço, maior comprimento do intestino delgado e menor pH duodenal para os leitões que receberam QOS em relação aos antibióticos. A morfometria indicou maior altura de vilosidades e melhor relação vilos/cripta para a colistina em relação ao QOS e menor escore lesional em relação aos demais tratamentos. A concentração sérica de imunoglobulinas apontou uma maior concentração de IgA para o QOS aos 35 dias. Frente aos resultados observados, embora o QOS tenha promovido estímulos sobre o sistema imune do animal, este não foi eficiente para substituir a colistina como promotor de crescimento para leitões desmamados com 17 dias de idade.
Palavras-chave: Colistina. Diarreia. Histologia. Imunoglobulinas. Lincomicina.

Introduction

Nowadays, one of the major problems in pork production in Brazil is the unrestricted use and abuse of antibiotics, intensifying the debate regarding food security and human health, especially the issue of antibiotic-resistant pathogens (MORÉS, 2014).

There are several products in the market listed as an alternative to antibiotic drugs as growth promoters; however, the results remain inconsistent and largely dependent on specific conditions. In recent years, Europe has been taking measures to completely transform pig-farming, especially production of piglets, which include management and preventive measures and optimization of the physiological conditions of piglets from birth to the weaning period, which, in conjunction with a nutrition based on reduction in disturbances and modulation of intestinal microbiota, serve as promising strategies for obtaining favorable results for the production of good-quality piglets in a sustainable way (HEO et al, 2013).

Among the additives currently studied and used by industry, prebiotics play a decisive role in the main strategies intended for better breeding conditions, which results in a constant search for new substances. To fulfill this need, Itano (2006) produced a chito-oligosaccharide as a by-product of the fermentation of silkworm pupae flour from the silk industry, with the purpose of evaluating its potential prebiotic activity in farm animals. This hypothesis is based on consistent results that other chito-oligosaccharides have demonstrated a potential to stimulate intestinal health and immunity, particularly in nursing piglets (XIAO et al., 2014; XIONG et al., 2015). Thus, this prebiotic may be a viable growth promoter replacement for the most commonly used antibiotics for piglets in Brazil.

The present study aimed to evaluate the response of weaned piglets to the chito-oligosaccharide against their response to two antibiotics with specific gram-positive and negative activity, lincomycin and colistin, respectively, with respect to performance and the immune and intestinal development of these animals.

Materials and Methods

Preparation of the inoculum

The inoculum was produced from a strain of *Bacillus subtilis* DP4, obtained from the microbiological acquis of the Department of Food Science and Technology (Departamento de Ciência e Tecnologia de Alimentos-DCTA) of the State University of Londrina. The growth medium was

prepared according to Itano (2006), containing 7.10% of silkworm pupae flour as the sole carbon source (acquired from the silk company Empresa de Fiação de Seda Bratac, Londrina, PR) (Table 1) and 2.84% peptone as the sole nitrogen source. The pH of the medium was adjusted to 9.60 with 30% NaOH solution and it was sterilized in an autoclave at 121°C and 1 atmosphere of pressure for 15 min. Subsequently, the *B. subtilis* inoculum was used to inoculate 150 mL of growth medium and was grown at a temperature of 31°C for 96 h, stirred at 110 rpm.

Table 1. Centesimal composition of silkworm pupae flour acquired from the company Fiação de Seda Bratac, Londrina, PR.

Crude protein (%)	52.66	Isoleucine (%)	2.18	Threonine (%)	2.62
Ether Extract (%)	27.17	Leucine (%)	3.75	Potassium (mg kg^{-1})	7643.09
Calcium (%)	0.11	Glutamic acid (%)	6.75	Magnesium (mg kg^{-1})	3266.75
Total phosphorus (%)	0.69	Lysine (%)	3.47	Manganese (mg kg^{-1})	nd*
Alanine (%)	2.82	Cystine (%)	0.62	Zinc (mg kg^{-1})	162.29
Arginine (%)	2.73	Methionine (%)	1.69	Iron (mg kg^{-1})	89.11
Aspartic acid (%)	5.88	Phenylalanine (%)	2.67	Copper (mg kg^{-1})	14.65
Glycine (%)	2.35	Tyrosine (%)	2.91	Cobalt (mg kg^{-1})	8.50

* no detected.
Source: Itano (2006).

Production of the chito-oligosaccharide

To produce the chito-oligosaccharide through fermentation, the medium described above was used, being used an inoculation of a 5% taken from the previously prepared inoculum and maintained under the same conditions. After the fermentation time (96 h), the fermented growth medium was mixed with commercial oyster flour at a 2:1 ratio (v/w) and then this mixture was kiln-dried with air circulation at 45°C for approximately 48 h. Subsequently, the product was ground for homogenization and stored in polyethylene bags (46 × 28 cm), sealed, and kept in cold storage at 5°C until use. The dry matter content and COS content of the dry product were determined to be 94.84% and 1.11 g/100 g, respectively.

The procedures used for inoculum preparation, fermentation, and final product (COS) preparation were based on the methodology adapted from Itano (2006).

Description of the experiment

This experiment was subjected to the assessment of the Ethics Committee on the Use of Animals at the State University of Londrina, and its development was approved (CEUA No. 18883.2012.52)

Ninety-six weaned Pen Ar Lan® piglets (48 barrows and 48 females) were used, with an average age of 17 days and a body weight of 5.33 ± 0.369 kg. The animals occupied 24 metallic suspended stalls (1.5 m^2) with ribbed plastic floor, equipped with a 250 W incandescent lamp for heating, a nursing nipple-type water trough, and chute-type feeders with four mouths. Minimum and maximum average temperatures of the nursing stall, recorded in the experimental period, were 23.8 ± 2.5°C and 29.4 ± 3.0°C, respectively.

The treatments comprised a group of piglets receiving a basal diet containing 100 ppm of chito-oligosaccharide (COS) as a test treatment,

a group a basal diet containing 40 ppm of colistin as the positive gram-negative control treatment, and a group on a basal diet containing 4.4 ppm of lincomycin as the positive gram-positive control treatment. A negative control treatment was not used owing to the limited number of experimental stalls and because the purpose of this study was to evaluate the difference between efficacy of COS and that of gram positive and negative antibiotics.

The nutritional requirements of the animals and the composition of the ingredients were based on the recommendations by Rostagno et al. (2011). The phases proposed for the experiment were the pre-initial I (from 17 to 35 days), pre-initial II (36 to 48 days), and initial (from 49 to 63 days). Food and water were provided ad libitum throughout the experimental period.

The basal diet was formulated with corn, soybean meal, whey powder, and vitamin and mineral supplement as ingredients, calculated to be isocaloric. In treatments other than the test treatment, the test product (COS) was replaced by an innocuous element (calcium carbonate) to adjust the calcium levels of the feed. This procedure was made because the COS had calcium carbonate as dilution base. The inclusion of COS was not taken into account for the formulation of the diets, owing to its low percentage. Corn was used as an ingredient to compose the basal formula for the different proportions of colistin, lincomycin, and COS as ingredients (Table 2).

Performance variables, weight gain, feed intake and feed conversion ratio, and diarrhea index were evaluated during the three experimental phases and at the end of the total experimental period. The incidence of diarrhea in the piglets was assessed daily by evaluating the fecal consistency, and recording the number of affected animals and their clinical symptoms. Stool consistency was classified according to Sobestiansky and Barcellos (2007): 1—liquid feces; 2—creamy feces; 3—pasty feces; 4—normal feces. For the assessment of the incidence of diarrhea, the animals were individually evaluated, but the experimental unit was the stall, wherein a positive stall was one with at least one animal with a score lower than 3.

For the evaluation of IgA, IgG, and IgM serum immunoglobulins of all animals, the first blood sample was collected at 21 days of age, before the vaccination for circovirus and mycoplasma. The second blood sample was obtained at 35 days.

For the weight assessment of the gastrointestinal tract and their annexes organs and for the morphometric assessment of the villi in the small intestine, a female animal was selected from each stall, at 35 days of age, totaling 24 animals, with an average weight of 6.86 ± 0.64 kg. The animal was chosen based on its live weight, using one with a weight that was closer to the average weight of the animals in each block. The sacrifice of the animals was preceded by electrical stunning followed by the bleeding of the major vessels of the neck. At this time, a second blood sample was collected for evaluation of IgA, IgG, and IgM serum immunoglobulins of all animals, those sacrificed and those that remained in the experiment, by puncturing the superior vena cava.

After the slaughter, the sacrificed animals were eviscerated to assess the relative weight of the stomach, spleen, liver, small intestine, and large intestine, length of the small intestine, and duodenal pH (pH of the duodenal juice collected directly from the intestinal fragment immediately after evisceration), checked using a potentiometer.

Then, tissue fragments were collected from the middle portion of the duodenum, jejunum, and ileum, approximately 3 cm in length, which were washed in a saline solution and cut across and lengthwise, to expose the intestinal lumen. After 24 h in the fixative solution (10% formaldehyde), the fragments were washed and immersed in 70% alcohol and stored at room temperature. The fragments were cut into approximately 0.5 cm long samples, dehydrated in alcohol, diaphanized in xylol, and embedded in

paraffin. Tissue sections, 5 µm thick, were obtained with American Optical microtome, and between one section and the subsequent one 30 sections were discarded (BERTOLETTO et al., 2008); four semi-seriated sections were arranged on one slide. The sections were stained with hematoxylin-eosin for histological assessment and with Alcian blue stain for goblet cell count.

Table 2. Calculated centesimal and nutritional composition of basal experimental feed for the periods, pre-initial I (17 to 35 days), pre-initial II (36 to 48 days) and initial (49 to 63 days).

		Pre-initial I	Pre-initial II	Initial
		Basal formula		
Corn	%	51.80	58.89	63.65
Soybean meal	%	18.00	21.00	29.00
Whole milk (powder)	%	12.00	7.00	2.00
Whey	%	8.00	3.00	0.00
Plasma	%	3.00	3.00	0.00
Bi-calcium phosphate 18%	%	2.38	2.00	1.72
Vegetable oil	%	2.00	2.00	1.80
Erythrocytes (powder)	%	1.00	1.00	0.00
Ground salt	%	0.50	0.30	0.42
Premix[1]	%	0.40	0.40	0.40
Zinc oxide 79.9%	%	0.20	0.20	0.00
Calcite limestone 38%	%	0.17	0.51	0.51
L-Lysine	%	0.25	0.30	0.25
L-Threonine	%	0.15	0.20	0.10
DL-Methionine	%	0.15	0.20	0.10
Copper sulfate 25%	%	0.00	0.00	0.05
		Nutritional Levels		
Crude protein	%	19.82	20.07	19.00
Ether extract	%	7.63	5.72	4.68
Crude fiber	%	2.15	2.50	3.03
Lactose	%	10.00	5.00	0.70
Total lysine	%	1.40	1.40	1.20
Digestible lysine	%	1.27	1.26	1.05
Digestible threonine	%	0.81	0.76	0.67
Digestible methionine	%	0.43	0.47	0.36
Zinc	mg kg^{-1}	1700	1700	153
Copper	mg kg^{-1}	12.00	12.00	150
Calcium	%	0.84	0.79	0.69
Total phosphorus	%	0.84	0.74	0.64

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Digestible phosphorus	%	0.65	0.55	0.44
Metabolizable energy	Kcal	3513	3500	3420

[1]Premix, assurance levels per kg of product: Choline: 75,000 mg kg^{-1}, Vitamin a: 50,000 UI, Vitamin D3: 75,000 UI Vitamin E: 9000 mg kg^{-1}, Vitamin K3: 975 mg kg^{-1}, Vitamin B1: 500 mg kg^{-1}, Vitamin B2: 1,200 mg kg^{-1}, Vitamin B6: 750 mg kg^{-1}, Vitamin B12: 8,000 mcg kg^{-1}, Niacin: 5,000 mg kg^{-1}, Pantothenic Acid: 3,000 mg kg^{-1}, Folic Acid: 500 mg kg^{-1}, Biotin: 20,000 mg kg^{-1}, Iron: 30,000 mg kg^{-1}, Copper: 3,000 mg kg^{-1}, Manganese: 17,500 mg kg^{-1}, Zinc: 30,000 mg kg^{-1}, Iodine: 200 mg kg^{-1}, Selenium: 150 mg kg^{-1}, Phytase: 25,000 U kg^{-1}.

[2]For the proposed treatments, 0.901% of COS was included for the COS treatment, whereas 0.901% of calcium carbonate was included for the treatments with antibiotics, to correct the calcium level of these feed rations. For the inclusion of 40 mg kg^{-1} of colistin and 4.4 mg kg^{-1} of lincomycin, these components were added to the feed without replacing the ingredients in the formulation.

A histological score was established to compare the histological changes, where a correlation was set between the type and the extent of the lesion and its degree of severity (or severity factor) (Table 3). The lesion score of the segment was obtained by multiplying the degree of severity of the lesion and its extension determined by intensity or frequency observed. The organ lesion score was obtained by adding the lesion scores. The degree of severity was determined as: 1—minor injuries; 2—moderate injuries; 3—severe injuries. The extent of each lesion (intensity or frequency observed) was evaluated in three fields per animal and the score was: 0—without lesion, 1—small extent (25% of the intestinal section affected), 2—intermediate extent (50% of the intestinal section affected), 3—large extent (75% of the intestinal section affected). Each intestinal section had an area of 1 cm^2 (BRACARENSE et al., 2012).

Table 3. Histological criteria used to establish the intestinal lesion score.

Type of lesion	Severity factor	Extent of the lesion	Maximum Score
Dilation of lymphatic vessels	1	0 to 3	38
Cell vacuolation	1	0 to 3	
Cuboidal epithelium (enterocytes)	2	0 to 3	
Flattening of villi	2	0 to 3	
Fusion of villi	2	0 to 3	
Interstitial edema	2	0 to 3	
Apical necrosis of villi	3	0 to 3	

The lesion score of the segment was obtained by multiplying the severity factor (or severity degree) of the lesion and its extension determined by the intensity or frequency observed. The organ lesion score was obtained by adding the lesion scores. The severity factor (or severity degree) was determined as: 1- minor injuries, 2 -moderate injuries, 3 -severe injuries. The extent of each lesion (intensity or frequency observed) was evaluated in three fields per animal and the score was: 0 - without lesion, 1- small extent (25% of the intestinal section affected), 2 -intermediate extent (50% of the intestinal section affected), 3 -large extent (75% of the intestinal section affected). Each intestinal section had an area of 1 cm^2.

Source: Bracarense et al. (2012).

The height and depth of villi were measured on 30 villi randomly chosen using the program MOTIC Image Plus 2.0 ML® image analysis system (MOTIC Image Plus Motic Instruments, Richmond, Canada). The number of lymphocytes, plasma cells, and eosinophils were counted in the region of the lamina propria in three random fields per sample, using the 40x objective. For goblet cell count, 30 random fields were chosen per sample. The averages of the lesion score, intestinal morphology, and number of goblet cells and inflammatory cells were used for statistical analysis, with at least 30 villi per segment being analyzed, and the averages of these villi treated as experimental units. Only the villi linked to the lamina propria and with well-defined ends were measured.

For the immunological analysis, sera were diluted for IgG (1:100,000), IgA, and IgM (1:10,000) quantitation, according to the manufacturer's instructions for Pig IgM, IgA, and IgG ELISA Quantitation Sets (Bethyl Laboratories, Inc.).

Study design and statistical analysis

The experimental design comprised randomized blocks (based on initial weight), with three treatments, eight repetitions and four animals per experimental unit (the stall) for the performance variables; for the other analyses, individual animals were used as an experimental unit. The gender of the animals was not taken into account for the statistical design as it had no influence on the intended analysis ($P > 0.05$); gender was used only to divide the experimental animals such that each stall contained two females and two males for each treatment.

The parametric data were subjected to variance analysis with the Tukey test, at 5% significance for difference in averages and at 10% significance to indicate a tendency of averages to differ, with the GLM package of the MINITAB 17 program. The data for incidence of diarrhea was analyzed by Chi-square of contingency as to the positivity of the animal for diarrhea, the incidence of watery diarrhea, and average fecal score during the period of 17 to 35 days. Non-parametric data of the immunological analysis underwent logarithmic transformation and were subsequently analyzed as parametric data.

Results and Discussion

Regarding growth performance (Table 4), during the initial phase (49 to 63 days), the antibiotic treatments showed a tendency for a higher daily weight gain (DWG) when compared to the 100 ppm COS treatment, promoting an increase of 100 g day^{-1} in the DWG ($P = 0.10$). The feed conversion ratio (FCR) of the Pre-initial phase I (17 to 35 days) was better ($P \leq 0.05$) for the piglets fed with colistin than those fed with COS and lincomycin. The incidence of diarrhea, observed in the pre-initial phase I (Table 5), was greater in animals fed with COS than those fed with antibiotics, and the COS treatment animals were also the most affected by watery feces (fecal score 1).

According to Gonzales, Mello and Café (2012), the tendency ($P = 0.10$) for a higher daily weight gain (DWG) response in the initial phase (49 to 63 days) under the antibiotic treatment than under the COS treatment may be associated with the antimicrobial action of these substances on intestinal microbiota, reducing the competition for nutrients and metabolite production that delay the growth of the animals. The best growth performance with antibiotics as growth promoters is commonly observed (PALERMO NETO; ALMEIDA, 2006; PARTANEN, 2002), as are their synergistic effects with other substances such as plasma (RODRIGUES, 2013) and copper sulfate (MENTEN, 1995), used as ingredients in the experimental diets in this study.

The higher feed conversion ratio (FCR) in the immediate post-weaning phase by use of colistin, when compared with the other treatments, may be associated to the lower incidence of diarrhea in this treatment, especially the watery diarrhea, observed in the same period. Although post-weaning diarrhea

has different etiologies, food handling and the digestive adaptability of the piglets to the diet offered are important factors affecting the incidence of post-weaning diarrhea syndrome (KUMMER et al., 2009), in addition to the effect of infectious agents. The low weaning age of 17 days, might affect the digestive adaptability of piglets for the first nursing diet and thus contribute to the higher incidence of watery diarrhea, which is mainly caused by colibacillosis (KYRIAKIS et al., 1999). In this sense, the colistin treatment was effective in reducing watery diarrhea, and its response relates to the activity spectrum of polymyxins E that act selectively on enteric gram-negative bacilli, particularly on *Escherichia coli*. The colibacillosis promotes colonization of the small intestine and releases enterotoxins that act on the functional barriers of the mucous membrane, increasing its permeability and leading to loss of fluids into the intestinal lumen, which characterizes the onset of watery diarrhea (GUIGNOT et al., 2007). However, in the present study, the cause of the diarrhea has not been identified.

Table 4. Zootechnical performance of piglets fed with colistin (COL), lincomycin (LIN), and chito-oligosaccharides (COS) for the different phases of nursery diet treatments.

Parameters	Treatments			Average	Average SE	P value
	COS	COL	LIN			
17 to 35 days (Pre-initial I)						
Initial weight (kg)	5.34	5.33	5.34	5.34	0.09	0.999
Final weight (kg)	6.71	7.22	6.63	6.86	0.16	0.162
DFI (g)	210	236	200	215	0.01	0.183
DWG (g)	80	112	76	89	0.01	0.135
FCR	2.63 B	2.10 A	2.63 B	2.41	0.12	0.047[1]
36 to 48 days (Pre-initial II)						
Final weight (kg)	12.86	12.91	12.32	12.36	0.26	0.267
DFI (g)	717	821	720	747	0.02	0.233
DWG (g)	381	416	403	400	0.01	0.558
FCR	1.88	1.87	1.78	1.87	0.04	0.263
49 to 63 days (Initial)						
Final weight (kg)	17.65	20.5	19.48	19.06	0.52	0.141
DFI (g)	942	968	906	939	0.02	0.576
DWG (g)	446 B	549 A	551 A	515	0.02	0.100[2]
FCR	2.11	1.76	1.64	1.82	0.06	0.133
17 to 63 days (Total period)						
DFI (g)	578	622	567	589	0.01	0.223
DWG (g)	280	335	321	312	0.01	0.157
FCR	2.06	1.85	1.76	1.88	0.07	0.164

DFI - Daily feed intake; DWG - Daily weight gain; FC - Feed conversion ratio.
[1]Different letters indicate statistically different values as per the Tukey test at 95% confidence level.
[2]Different letters indicate statistically different values as per the Tukey test at 90% confidence level.

Table 5. Incidence of diarrhea and watery diarrhea and average fecal score index for piglets fed with 100 ppm of chito-oligosaccharides (COS), 40 ppm of colistin (COL), or 4.4 ppm of lincomycin (LIN) during the pre-initial I phase (from 17 to 35 days).

Parameters	Treatments			P value
	COS	COL	LIN	
17 to 35 days				
Incidence of diarrhea (%)[1]	100 B	50 A	83.33 AB	0.046
Incidence of watery diarrhea (%)[1]	100 C	0 A	50 B	0.002
Average fecal score[2]	3.3 B	4.0 A	3.7 AB	0.016

[1]Chi-square contingency test. For different letters, numbers statistically different at $P < 0.05$.
[2]Assessment of fecal score, assuming the average frequency of the score for normal feces as normal. Different letters indicate statistically different values at $P < 0.05$.

Colistin has a bactericidal action on these pathogens, reducing their population in the intestinal microbiota and thus favoring the operational integrity of the proteins that constitute the occluding junctions (*tight junctions*), the occlusion zonules, occludins, and claudins (SUZUKI, 2013), thereby reducing the membrane permeability and consequently, the incidence of watery diarrhea.

Yang et al. (2012) observed no differences in the *E. coli* count for the cecum content, with feed rations including 20 ppm of colistin and 200, 400, and 600 ppm of COS during 14 days after weaning, and the COS levels of 400 and 600 ppm in the feed also showed similar DWG and FC as those obtained with feed containing antibiotics. In this sense, the level of 100 ppm of COS in the experimental diet used in the present study, four to six times lower than the one used in previous studies, may not have been enough to demonstrate benefits within the first 14 post-weaning days, when compared with the antibiotics colistin and lincomycin.

Similar to the animals fed with colistin, the animals under the lincomycin treatment presented a trend of higher DWG than those under the COS treatment, in the initial phase of the experiment; however, the animals that received lincomycin showed worse results ($P < 0.050$) for feed conversion ratio and incidence of watery diarrhea than the animals fed with colistin. Lincomycin is an active lincosamide against gram-positive bacteria, mycoplasma, and some anaerobic bacteria, with a restricted activity against gram-negative aerobic bacteria such as enterotoxigenic *E. coli* (SPINOSA, 2006).

The relative weight of the digestive tract organs (Table 6) showed a relative increase in the spleen weight ($P \leq 0.05$) for animals fed with 100 ppm of COS compared with that in animals fed with antibiotics; the duodenal pH was lower ($P \leq 0.05$) in animals fed with COS than in animals fed with colistin. The antibiotic treatments had a significant effect on the length of the small intestine ($P \leq 0.05$) and the animals in the two antibiotic groups (gram-positive and gram-negative) showed a longer small intestine than the animals under the COS treatment.

Table 6. Relative weights of stomach, liver, spleen, small intestine, and large intestine (% of final weight at slaughter) and length of the small intestine of 35-day-old piglets fed with rations with 100 ppm of chito-oligosaccharides (COS), 40 ppm of colistin (COL), or 4.4 ppm of lincomycin (LIN).

Parameters	Treatments			Average	Average SE	P value
	COS	COL	LIN			
Organ Weight						
Stomach (%)	0.77	0.73	0.88	0.79	0.04	0.198
Liver (%)	2.72	2.46	2.56	2.58	0.07	0.285
Spleen (%)	0.32 A	0.20 B	0.19 B	0.24	0.04	0.050[1]
Small intestine (%)	4.24	4.54	4.44	4.41	0.14	0.212
Large intestine (%)	2.17	2.58	2.38	2.38	0.11	0.326
Small intestine						
Length (m)	7.22 B	8.53 A	8.00 A	7.92	0.30	0.031[1]
Relative length	0.46 B	0.55 A	0.54 A	0.516	0.04	0.055[2]
pH of the duodenum	6.55 B	7.11 A	6.75 A	6.80	0.13	0.007[1]

[1]Different letters indicate statistically different values as per the Tukey test at 95% confidence level.
[2]Different letters indicate statistically different values as per the Tukey test at 90% confidence level.

As to the visceral assessment, the smallest length of the small intestine (total and relative) may be related to the high incidence of watery diarrhea and villous atrophy that reduce the time for recovery and development of the intestinal epithelium (LALLÉS et al., 2004); although the rate of cell replication of the crypts is a response to tissues aggression, it is not a positive response of the development of the intestinal epithelium (MONTAGNE; PLUSKE; HAMPSON, 2003). Andrade et al. (2011) found increased intestinal length with the use of colistin while evaluating different nucleotide sources, whereas Rizzo et al. (2010) and Costa, Tse and Miyata (2007) observed a reduction in the intestinal length with the use of antibiotics in chickens and pigs, respectively, and attributed this response to an improvement in intestinal health of the animals, because an increase in intestinal length could result in greater energy expenditure and lower feed efficiency for the animal (GOMES et al., 2007). Comparing the average relative length of the small intestine reported in other studies, it was found that the results for this parameter in the present study were lower than those reported in other studies (0.516 μm versus 0.950 μm, according to Andrade et al. (2011) and 0.720 μm, as noted by Costa et al. (2011), thus demonstrating that there was some setback in the development of the small intestine for all treatments, with the COS treatment showing the lowest relative length.

Regarding the relative spleen weight, Andrade et al. (2011), when working with nucleotides, attributed the increase in relative spleen weight to a possible stimulus to immunity in recently weaned piglets. For COS, Xiong et al. (2015) determined that, at low dosages (30 ppm), COS can stimulate an immune response at the mucous membrane level. However, for higher levels (300 mg kg^{-1}), Xiao et al. (2014) identified that COS was effective to relieve the intestinal inflammatory process and stimulate the cell-mediated immune response. However, an increase in relative spleen weight may also be associated with hypersensitivity processes in the gastrointestinal tract and stimuli of invasion of this mucous membrane by opportunistic pathogens, such as enterotoxigenic *Escherichia coli*, which

may result in severe diarrhea, as was observed in this experiment for animals that received COS (ALTAMURA et al., 2001).

For the duodenal pH, normal values are between 4 and 6 for piglets; however, the closer the pH to 4, the better the piglets' response will be to the proliferation of pathogens in the duodenum, because the optimal pH values for bacterial growth are above 6: pH 6-8 for *Escherichia coli*, 6-7.5 for *Streptococcus* sp. and *Salmonella*, 6.8-7.5 for *Staphylococcus* sp. and 6.6-7.0 for *Pseudomonas* (RAVINDRAN; KORNEGAY, 1993).

In this experiment, the pH values for piglets fed with COS were lower than those for piglets under the other treatments; however, they were above the normal value of 6.55. This value can indicate, among other things, a stopper effect of the base feed (from calcium carbonate and limestone), in addition to a low acidifying effect of the diet due to the action of lactose and lack of organic acids as additives for stomach pH reduction (KORNEGAY; EVANS; RAVINDRAN, 1994). The results found by Xiong et al. (2015), however, suggest a higher stomach pH value with the use of COS in low dosage (30 ppm), but there was no difference in the duodenal pH when compared with the use of antibiotics (chlortetracycline). Some studies in patients with inflammatory bowel disease and severe lesions of the duodenum indicate a reduction in the pH of that segment, probably because of the low sodium bicarbonate excretion (VERNIA et al., 1988; NUGENTS et al., 2001).

Morphometric evaluation of the different portions of the small intestine (Table 7) revealed a greater villus height ($P \leq 0.05$) and a higher ratio of villus height to crypt depth in the jejunum with 40 ppm of colistin in the diet than with 100 ppm of COS. The colistin treatment resulted in higher villus height and consequently, a higher ratio of villus height to crypt depth, than the COS treatment. These results differ from those found by Yang et al. (2012), in which data did not differ between animals fed with 20 ppm of colistin and animals fed with different COS levels (200, 400 and 600 ppm).

Table 7. Morphological characteristics of the small intestine of 35-day-old piglets fed with rations with 100 ppm of chito-oligosaccharides (COS), 40 ppm of colistin (COL), or 4.4 ppm of lincomycin (LIN).

Parameters	Treatments			Average	Average SE	P value
	COS	COL	LIN			
			Duodenum			
Villi (µm)	165	174	164	168	8.2	0.86
Crypt (µm)	214	230	225	223	7.91	0.78
Goblet cells (n)	4.9	5.1	4.1	4.7	0.35	0.59
Villus/crypt ratio	0.77	0.76	0.74	0.76	0.03	0.96
			Jejunum			
Villi (µm)	149 B	203 A	174 AB	175	8.32	0.02[1]
Crypt (µm)	186	204	205	198	7.41	0.59
Goblet cells (n)	3.8	4.3	3.5	3.9	0.2	0.30
Villus/crypt ratio	0.81 B	1.00 A	0.86 AB	0.89	0.03	0.03[1]

Continue...

Continuation...

			Ileum			
Villi (µm)	156	172	156	161	5.22	0.40
Crypt (µm)	171	201	180	184	8.08	0.40
Goblet Cells (n)	6.8	5.9	5.8	6.2	0.49	0.69
Villus/crypt ratio	0.92	0.87	0.90	0.90	0.03	0.88

[1]Different letters indicate statistically different values as per the Tukey test at 95% confidence level.

Depletion in villus height and mainly in the ratio of villus height to crypt depth are efficient criteria for assessing the digestibility of nutrients from the intestinal epithelium in response to the aggression on tissues caused by physical, chemical, and/or microbiological agents during the post-weaning period, especially in early weaning, and these tend to become normal after 5 to 10 days post weaning (LALLÉS et al., 2004; MONTAGNE; PLUSKE; HAMPSON, 2003). In this sense, the lower incidence of watery diarrhea in animals treated with colistin than in those treated with COS is likely associated with the higher villus height and the highest ratio of villus height to crypt depth. The mechanisms responsible for the best intestinal morphology must also be associated with the ability of colistin in controlling the population of pathogenic gram-negative bacteria, especially *E. coli*, and consequently, reduce their harmful action on the intestinal mucosa.

Greater villus height and its ratio to crypt depth are important parameters in the assessment of intestinal health and tissue recovery rate of this organ, indicating a better functioning, especially for nutrient absorption, of the intestinal segments and especially the jejunum (LALLÉS et al., 2004). In the present study, these features were more favorable for the animals subjected to the 40 ppm colistin treatment than for the animals that received 100 ppm of COS.

The higher incidence of lesions for the use of COS in the jejunum portion was characterized by a more pronounced cuboidal epithelium for 150 mg $Kg^{-1}$and fusion of villi with greater lesion score for 100 ppm of COS. For the ileum, the highest incidence of lesions was also characterized by cuboidal epithelium and fusion of villi, with a more pronounced lesion score for the animals in the control group than in other test groups (Figure 2). Also for the somatic cell count (SCC), results indicate a greater number of lymphocytes in the lamina propria ($P \leq 0.05$) of the jejunum and ileum in piglets fed with COS than in those fed with colistin and lincomycin, respectively (Table 8). Piglets fed with colistin also presented fewer lymphocytes ($P \leq 0.05$) in the jejunum than those fed with lincomycin, showing that in the animals under colistin treatment, there was not such a severe aggression with a consequent increase in epithelial lesions and increase in the inflammatory process, when compared to animals fed with COS and lincomycin.

Table 8. Somatic cell count and the differentiation of somatic cells in the small intestine of 35-day-old piglets fed with rations with 100 ppm of chito-oligosaccharides (COS), 40 ppm of colistin (COL), or 4.4 ppm of lincomycin (LIN).

Parameters	Treatments			Average	Average SE	P value
	COS	COL	LIN			
Duodenum						
SCC (n)	90.5	88.1	78.7	85.8	4.6	0.54
Lymphocytes (n)	36.1	29.6	37.1	34.3	1.9	0.22
Plasma cells (n)	40.8	32.2	30.7	34.6	4.0	0.44
Eosinophils (n)	13.6	26.3	10.9	13.6	1.5	0.39
Jejunum						
SCC (n)	77.5	68.2	66.9	70.9	3.4	0.46
Lymphocytes (n)	41.4 A	30.5 B	40.2 A	37.4	2.0	0.05[1]
Plasma cells (n)	13.9	15.1	8.9	12.6	1.7	0.29
Eosinophils (n)	22.2	22.5	17.2	20.6	1.7	0.50
Ileum						
SCC (n)	90.1	85.0	79.4	84.8	4.0	0.61
Lymphocytes (n)	40.5 A	33.3 AB	30.4 B	34.7	2.0	0.04[1]
Plasma cells (n)	6.9	9.9	7.1	8.0	0.7	0.15
Eosinophils (n)	44.7	41.8	41.9	42.8	3.9	0.99

SCC - Somatic cell count
[1]Different letters indicate statistically different values as per the Tukey test at 95% confidence level.

The presence of lymphocytes in the lamina propria of the jejunum showed results similar to those obtained by Xiong et al. (2015), who also demonstrated this effect in the lamina propria of the jejunum and duodenum using COS. These authors, however, observed a reduction in lymphocyte number in the lamina propria of the ileum with the use of COS, in relation to a negative control.

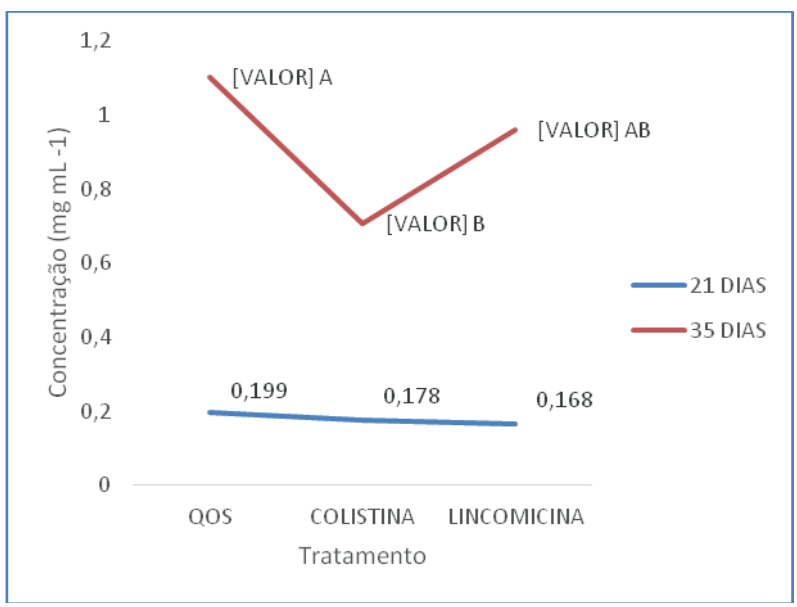
The serum immune response (Table 9) indicated a trend ($P < 0.10$) of increase in serum IgA for 35-day-old piglets treated with COS versus colistin (Figure 1) which may be associated with the use of COS as a growth promoter. Ma et al. (2014) observed an increase in the serum IgA of animals fed with 100 ppm of COS associated with chelated Zn, but there was no increase in serum concentrations of IgM and IgG, similar to the results of the present study. Xiong et al. (2015) also observed increased concentration of IgA in animals fed with COS in relation to the negative control group. Yin et al. (2008), comparing 250 ppm of COS with 110 ppm of lincomycin for piglets at weaning, observed an increase in IgA, IgG, or IgM, in addition to the interleukins IL1, IL2, and IL6, suggesting that COS probably played a fundamental role in the promoting cell-mediated immune response via modulation in cytokine and antibody production. These results demonstrate that the prebiotic COS presented better outcomes for growth and animal health than those observed with lincomycin.

Table 9. Serum immune response (Total, IgG, IgA, and IgM) of 21- and 35-day-old piglets fed with 100 ppm of chito-oligosaccharides (COS), 40 ppm of colistin (COL) or 4.4 ppm of lincomycin (LIN).

Parameters	Treatments			Average	Average SE	P value
	COS	COL	LIN			
21 days						
Total Ig (mg mL^{-1})	10.7	11.1	10.7	10.8	0.43	0.80
IgG (mg mL^{-1})	9.1	9.5	9.1	9.2	0.38	0.26
IgA (mg mL^{-1})	0.199	0.178	0.168	0.182	0.01	0.11
IgM (mg mL^{-1})	1.41	1.46	1.39	1.42	0.11	0.96
35 days						
Total Ig (mg mL^{-1})	12.5	14.1	13.5	13.4	0.47	0.50
IgG (mg mL^{-1})	8.5	10.1	9.2	9.3	0.40	0.86
IgA (mg mL^{-1})	1.103 A	0.709 B	0.962 AB	0.925	0.07	0.07[1]
IgM (mg mL^{-1})	2.85	3.29	3.36	3.17	0.17	0.43

[1]Different letters indicate a tendency to statistically different averages as per the Tukey test at 90% confidence level, with logarithmic transformation of non-parametric data.

Figure 1. Serum immune response of IgA (in mg mL^{-1}) of 21- and 35-day-old piglets fed with rations with 100 ppm of chito-oligosaccharides (COS), 40 ppm of colistin (COL), or 4.4 ppm of lincomycin (LIN).



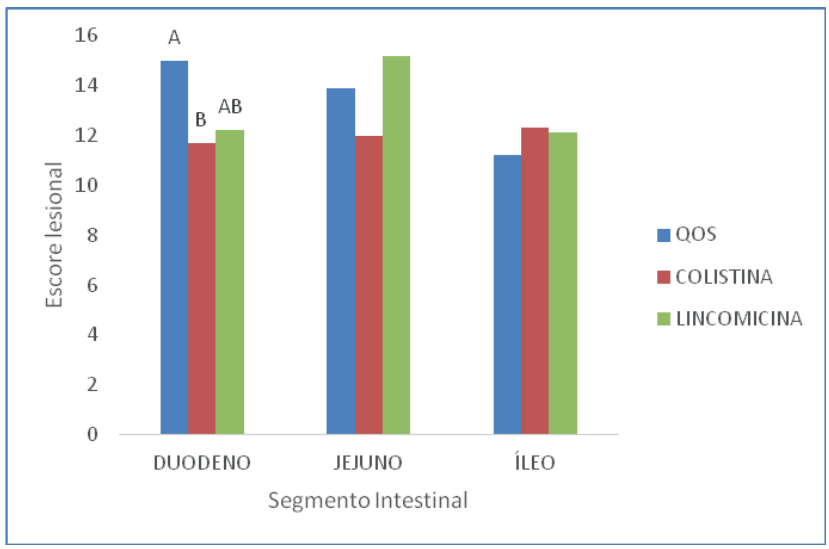
Translation of the captions in Figure 1
Concentração = Concentration (mg mL^{-1})
QOS = COS
COLISTINA = COLISTIN
LINCOMICINA = LINCOMYCIN
Tratamento = Treatment
21 DIAS = 21 DAYS
35 DIAS = 35 DAYS

[1] Different letters indicate tendency to statistically different averages ($P < 0.10$) as per the Tukey test, with logarithmic transformation of non-parametric data.

For epithelial lesions, it was observed that piglets fed with 100 ppm of COS showed a tendency ($P \leq 0.10$) to present a greater score of epithelial lesions of the duodenum than piglets fed with 40 ppm of colistin (Figure 2). However, the epithelial lesion response of the ileum was graphically lower for the

COS treatment, corroborating the results obtained by Li et al. (2013) and Xiong et al. (2015); however, in the present study, there was no statistically significant difference between treatments for the ileum.

Figure 2. Epithelial lesion score for different segments of the small intestine of piglets fed with rations with100 ppm of chito-oligosaccharides (COS), 40 ppm of colistin (COL) or 4.4 ppm of lincomycin (LIN).



Translation of the captions in Figure 2
Escore lesional = Lesion Score
QOS = COS
COLISTINA = COLISTIN
LINCOMICINA = LINCOMYCIN
DUODENO = DUODENUM
JEJUNUM = JEJUNUM
ÍLEO = ILEUM
Segmento intestinal = Intestinal segment

*Score of epithelial lesions after histological examination according to the frequency and severity of lesions in the small intestine according to Bracarense et al. (2012). [1]Different letters indicate statistically different averages for the same intestinal segment ($P < 0.10$) as per the Tukey test.

Thus, the results of DWG, FCR, incidence of diarrhea, score of epithelium lesions, and intestinal morphology observed in this study differ from those obtained previously by some authors who worked with colistin as a reference antibiotic (YANG et al., 2012) for the use of COS or even when this was compared to a negative control, indicating many positive aspects of the use of this type of oligosaccharide (HAN et al., 2007; XIONG et al., 2015; YANG et al., 2012).

Conclusion

Colistin presented consistent responses on the growth performance, incidence of diarrhea, and intestinal morphology in relation to the treatments with lincomycin and COS in piglets under the pre-existing adverse conditions of weaning initiated at 17 days of life.

COS was not effective as a single-effect replacement for lincomycin and colistin as growth promoters for the parameters studied, under the experimental conditions used. The consistent immunostimulation response mediated by COS versus the antibiotics evaluated, suggests an effect on the modulation of the immune system that should be explored at critical periods such as weaning, necessitating further investigation.

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