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Chitooligosaccharide for piglets: effects on performance, viscera and intestinal morphometry

Quitooligossacarídeo para leitões: efeitos sobre a performance, órgãos viscerais e morfometria intestinal

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Abstract

The objective of this study was to evaluate the dose-response effect of a chitooligosaccharide (COS) produced by Universidade Estadual de Londrina over performance, diarrhea episodes, visceral characteristics and morphometry. A total of 72 Pen Ar Lan[®] piglets were weaned at 23 days and 7.51 ± 1.35 kg of BW. The treatment was a basic diet plus inclusion of 0, 50, 100, or 150 ppm of COS (100 mg/kg). For the visceral and morphometric evaluation, 24 animals were slaughtered at 31 days of age with 11.01 ± 1.25 kg of BW. No regression effect over the performance or diarrhea prevalence was observed. Higher doses of COS led to higher absolute values for large intestine weight, villus height in the jejunum and crypt depth in the jejunum and ileum; the villus/crypt ratio in the ileum was higher until the level of 100 ppm COS. More lesions were observed in the intestinal epithelium along with a higher total number of lymphocytes and eosinophils in the duodenum. High numbers of eosinophils were observed in the ileum when a higher level of COS was used. In general, increasing levels of COS led to better intestinal morphometry and activated somatic cells in the lamina propria of the piglet intestine; however, no influence was observed in the productive performance of these animals. Considering all studied parameters, the COS level of 100 ppm was the most promising under these experimental conditions.

Key words: Nursery. Diarrhea. Prebiotic. Performance. Alternative.

Resumo

Objetivou-se com este trabalho avaliar o efeito dose-resposta do Quitooligossacarídeo (QOS) produzido pela Universidade Estadual de Londrina sobre o desempenho de leitões em creche e morfohistometria

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e peso de órgãos de leitões aos 31 dias de idade. Foram utilizados 72 leitões de genética Pen Ar Lan®, desmamados com idade média inicial de 23 dias e peso de $7,51 \pm 1,35$ kg. Os tratamentos consistiram em uma dieta base com os níveis de inclusão de 0, 50, 100 e 150 ppm de QOS. Para a avaliação visceral e morfohistométrica, 24 animais foram abatidos aos 31 dias de idade e peso de $11,01 \pm 1,25$ kg. Não houve efeito de regressão sobre o desempenho ou incidência de diarreia. O aumento da dose utilizada de QOS levou a um aumento nos valores referentes ao peso relativo do intestino grosso, à altura de vilosidades de jejuno e à profundidade de cripta no jejuno e íleo, enquanto que, a relação vilos/crípta deste último segmento foi crescente apenas até o uso de 100 ppm de QOS. Observou-se aumento de lesões no epitélio intestinal e contagem de linfócitos e eosinófilos no duodeno e eosinófilos também no íleo com o aumento da dose do QOS até 150 ppm. De maneira geral, os níveis crescentes de QOS melhoraram a morfofisiologia intestinal e ativaram a células somáticas na lâmina própria do intestino dos leitões, entretanto, não houve influência sobre o desempenho produtivo dos animais. Frente a todos os parâmetros estudados, o nível de 100 ppm de QOS mostrou-se o mais promissor para as condições propostas por este experimento.

Palavras-chave: Creche. Diarreia. Prebiótico. Performance. Alternativa.

Introduction

The indiscriminate usage of antibiotics in animal production can grow pathogen selectivity (BURCH, 2005; MORÉS, 2014), directly disrupting the efficiency of these products for human health. Due to this, in global swine production, especially in Europe, viable alternatives for antibiotics are sought that do not impose a severe reduction in performance and sanitary conditions.

Prebiotics are substances used in swine production because they have a huge impact during the most critical period for these animals, i.e. weaning (ROBERFROID et al., 2010). Mannooligosaccharides and fructooligosaccharides are the most studied products for this category; however, new prebiotics, like chitooligosaccharides (COS) from chitosan have appeared as viable alternatives. In Brazil, however, there have been few studies using this product in animal production. COS is produced as a byproduct of the silk industry, i.e. chrysalid meal (ITANO, 2006). It has been shown to have promising effects in broilers (QUINTANA, 2009), and has been investigated in piglet trials, related to health status and immune system modulation, since prebiotics modulate interleukin activation, higher concentration in animal blood serum, increase the immune response and reduce

the inflammatory process in the intestine (HAN et al., 2007; MA et al., 2014; YIN et al., 2008).

The objective of this work, through a dose-response study, was to evaluate the prebiotic action of COS in weaning piglets at 23 days in terms of nursery performance, intestinal morphometry, and the presence and differentiation of somatic cells in lamina propria at 31 days of age.

Material

Inoculum production for chitooligosaccharide production

The inoculum was produced by *Bacillus subtilis* DP4, obtained from the Departamento de Ciência e Tecnologia de Alimentos da Universidade Estadual de Londrina (DCTA) microbiologic collection and used to inoculate 150 mL of prepared culture, according to Itano (2006), with 7.10% chrysalid meal as the only carbon source (Fiação de Seda BRATAC, Londrina-PR) (Table 1) and 2.84% peptone as an additional source of nitrogen. The pH was adjusted to 9.60 with 30% NaOH solution, and the medium was sterilized at 121°C, 1 atm during 15 minutes, followed by cultivation at 31°C for 96 hours with 110 rpm shaking.

Table 1. Centesimal composition of the chrysalis flour used in this study and in the previous works with the product in the State University of Londrina and obtained from the company Siao Bratac - Londrina - PR.

| Nutrient | | Nutritional Levels |
|---------------|-------|--------------------|
| Crude protein | % | 52.66 |
| Ether Extract | % | 27.17 |
| Calcium | % | 0.11 |
| Total match | % | 0.69 |
| Arginine | % | 2.73 |
| Isoleucine | % | 2.18 |
| Leucine | % | 3.75 |
| Glutamic Acid | % | 6.75 |
| Lysine | % | 3.47 |
| Cysteine | % | 0.62 |
| Methionine | % | 1.69 |
| Phenylalanine | % | 2.67 |
| Tyrosine | % | 2.91 |
| Threonine | % | 2.62 |
| Potassium | mg/kg | 7643.09 |
| Magnesium | mg/kg | 3266.75 |
| Manganese | mg/kg | nd |
| Zinc | mg/kg | 162.29 |
| Iron | mg/kg | 89.11 |
| Copper | mg/kg | 14.65 |
| Crude Protein | mg/kg | 8.50 |

Inoculum preparation of the fermented and final product (COS) used in this study was based on a method adapted from Itano (2006).

Chitooligosaccharide fermented preparation

For the preparation of the richest fermented COS, the environment was the same as before, and was inoculated with 5% of the previous inoculum under the same conditions. After 96 hours, the fermented culture was mixed with commercial oyster meal in a 2:1 (v/p) proportion and then dried in a kiln with air circulation at 45°C for 48 hours. Next, the product was crushed for homogenization and storage in polyethylene bags, then stored in a cold room at 5°C until its utilization. The dry matter content and COS level were 94.84% and 1.11 g/100 g, respectively.

Trial

The experiment was submitted to Comitê de Ética no Uso de Animais da Universidade Estadual de Londrina for ethical approval (CEUA 1883.2012.52).

In total, 72 piglets of the Pen Ar Lan® strain were used, weaned at 23.4 days old with an average BW of 7.51 ± 1.35 kg. The animals were placed in 24 suspended metallic pens, with 1.5 m² of space

and a ripped plastic floor, equipped with 250 W incandescent lamps for heat, a nipple drinker and a four-place feeder.

The min and max average temperatures registered during the experiment were $21.16 \pm 1.20^{\circ}\text{C}$ and $29.71 \pm 2.44^{\circ}\text{C}$, respectively. The relative air humidity during morning and afternoon was $78.25 \pm 6.0\%$ and $61.20 \pm 10.33\%$, respectively.

The experiment was a dose-response study of COS usage, with all animals distributed in full blocks design (per weaning weight) with the doses 0, 50, 100 and 150 ppm in six replicates and three animals per experimental unit; this was related to pens for performance and to animals for other analyses.

The animal nutritional requirements and the feed composition were based on Rostagno et al. (2011) (Table 2). The proposed phases were pre-starter 1 (23 to 35 days), pre-starter 2 (35 to 50 days), and starter (50 to 63 days). Feed and water were available ad libitum for the whole trial.

The growth, feed intake, feed conversion ratio and diarrhea score variables were evaluated during all phases. The diarrhea observation was done daily by an observer evaluating the consistency of feces, and the number of animals with diarrhea was noted. The consistency of feces was evaluated according to the scale of Sobestiansky and Barcellos (2007): 1 - liquid; 2 - creamy; 3 - pasty; 4 - normal. For data analysis, the pen was considered the experimental

unit and the pen was considered positive when at least one animal was considered positive.

Table 2. Pre-start I (23 to 35 days), pre-start II (36 to 50 days) and Starter (51 to 63 days) composition of experimental rations for the proposed periods.

| | | Pre-start I | | | | Pre-start II | Starter |
|---------------------------|---|-------------|------|------|------|--------------|---------|
| Levels of COS (ppm) | | 0 | 50 | 100 | 150 | | |
| | | Macro | | | | | |
| Corn | % | 52.0 | 52.0 | 52.0 | 52.0 | 52.7 | 60.5 |
| Soybean meal | % | 18.0 | 18.0 | 18.0 | 18.0 | 20.0 | 31.0 |
| Skim powdered milk | % | 17.2 | 17.2 | 17.2 | 17.2 | 16.3 | 0.00 |
| Plasma | % | 3.00 | 3.00 | 3.00 | 3.00 | 2.00 | 0.00 |
| Dehydrated Hemacytes | % | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 |
| Refined Vegetable Oil | % | 2.00 | 2.00 | 2.00 | 2.00 | 1.43 | 1.40 |
| Whey | % | 1.87 | 1.87 | 1.87 | 1.87 | 2.55 | 2.54 |
| Corn Gluten 60 | % | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 | 0.00 |
| Calcitic Limestone 38% | % | 0.80 | 0.80 | 0.80 | 0.80 | 0.80 | 0.70 |
| Dicalcium Phosphate 18.5% | % | 0.79 | 0.79 | 0.79 | 0.79 | 0.84 | 1.50 |
| Ground salt | % | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Premix ¹ | % | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 |

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| | | | | | | | |
|---|-------------|-------|-------|-------|-------|------------------------|------|
| Copper Sulfate 25% | % | 0.00 | 0.00 | 0.00 | 0.00 | 0.07 | 0.07 |
| L-Lysine HCl 79% | % | 0.07 | 0.07 | 0.07 | 0.07 | 0.04 | 0.02 |
| Relation COS/Calcium Carbonate | | | | | | | |
| COS | % | 0.000 | 0.450 | 0.920 | 1.370 | Same as previous phase | |
| Calcium Carbonate | % | 1.370 | 0.920 | 0.450 | 0.000 | | |
| Nutritional Levels | | | | | | | |
| Crude protein | % | 20.8 | 20.8 | 20.8 | 20.8 | 20.6 | 19.3 |
| Ether Extract | % | 7.50 | 7.50 | 7.50 | 7.50 | 6.90 | 3.83 |
| Crude Fiber | % | 2.45 | 2.45 | 2.45 | 2.45 | 2.30 | 3.09 |
| Humidity | % | 9.91 | 9.91 | 9.91 | 9.91 | 10.48 | 11.3 |
| Lactose | % | 7.35 | 7.35 | 7.35 | 7.35 | 7.80 | 1.80 |
| Apparent digestible lysine | % | 1.20 | 1.20 | 1.20 | 1.20 | 1.14 | 1.03 |
| Apparent digestible threonine | % | 0.78 | 0.78 | 0.78 | 0.78 | 0.74 | 0.67 |
| Apparent digestible methionine/ cysteine | % | 0.72 | 0.72 | 0.72 | 0.72 | 0.68 | 0.62 |
| Apparent digestible tryptophan | % | 0.25 | 0.25 | 0.25 | 0.25 | 0.24 | 0.22 |
| | | | | | | | |
| Calcium | % | 0.94 | 0.94 | 0.94 | 0.94 | 0.89 | 0.71 |
| Digestible Phosphorus | % | 0.44 | 0.44 | 0.44 | 0.44 | 0.43 | 0.41 |
| Metabolizable Energy | Kcal/ kg | 3613 | 3613 | 3613 | 3613 | 3618 | 3349 |

*COS: chitooligosaccharide preparation (1,11%).

¹. Requirement levels for 1 kg of Premix: Coline: 75,000 mg kg⁻¹, Vitamin A: 50,000 UI, Vitamin D3: 75,000 UI, Vitamin E: 9,000 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, Pantotenic 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, Selene: 150 mg kg⁻¹, Phytase: 25,000 U kg⁻¹.

For the evaluation of organ weight and gastrointestinal tract morphometry, one female animal per pen was used at 31 days of age, with 24 animals evaluated with an average weight of 11.0 kg. The chosen animal was determined by the live weight and the cull was preceded by electric shock and large vessel bloodletting. After slaughter, the animals were gutted and the contents removed to determine the relative empty weight of the stomach,

were collected. The intestinal fragments, 3 cm in length, were washed in physiological solution and then sectioned transversally and longitudinally to expose the intestinal lumen. After 24 hours of fixation (0% formol) sections were stored in 70% alcohol at room temperature. The fragments were cut into 0.5 cm long samples, dehydrated in alcohol, diaphanous in xylol and embedded in paraffin. Each block was sectioned in four semi-serial sections at a depth of 5 µm, obtained with an American Optimal microtome, with 30 sections discarded between sections (BERTOLETTO, 2005). The 5 µm thick section were stained with hematoxylin and eosin for histological evaluation and with Alcian Blue for the analysis of goblet cells. The histological evaluation compared changes assessed correlations between the type and extensions of the lesions and

its severity (Table 3). The lesion score was obtained for the severity factor multiplication (or severity degrees) of the lesion and its extension was determined by its intensity or observed frequency. The lesion score of this organ was obtained by summing the scores. The severity degree was determined as: 1 - mild; 2 - moderate; 3 - severe. The extension of each lesion was observed in three areas per animal and scored as: 0, nothing; 1, small extension (25%), 2, intermediate extension (50%); 3, large extension (75%). Each section had an area of 1 cm² (BRACARENSE et al., 2012).

Table 3. Histological criteria used to establish the score of intestinal lesions.

| Lesion | Severity Factor | Lesion Extension | Max score |
|---------------------------------|-----------------|------------------|-----------|
| Dilatation of lymphatic vessels | 1 | 0 a 3 | 38 |
| Cellular vacuolization | 1 | 0 a 3 | |
| Cubic epithelium (enterocytes) | 2 | 0 a 3 | |
| Villous scaling | 2 | 0 a 3 | |
| Fusion of villi | 2 | 0 a 3 | |
| Interstitial edema | 2 | 0 a 3 | |

Apical villi necrosis

3

The lesion score of the segment was obtained by multiplying the severity factor (or degree of severity) of the lesion and its extent determined by the intensity or frequency observed. The lesion score of the organ was obtained by adding the lesion scores. The severity factor (or degree of severity) was determined as: 1 - mild lesions; 2 - moderate lesions; 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 extension (25% of affected intestinal section); 2, intermediate extension (50% of affected intestinal section); 3, large extension (75% of affected intestinal section). Each intestinal section was 1 cm².

Source: Bracarense et al. (2012).

The villi height and crypt depth were measured in 30 villi chosen randomly using the MOTIC Image Plus 2.0 ML[®] image analysis system (MOTIC Image Plus Motic Instruments, Richmond, Canada). The number of lymphocytes, plasmolytic cells, and eosinophils was counted in the region of lamina propria, in three randomly fields per sample with a 40x objective. For goblet cells, 30 fields were randomly chosen per sample. The lesion score average, intestinal morphometry, goblet cell numbers, and inflammatory cells were used in the statistical analysis with 30 fields per segment and

the villus average as the experimental unit. Only villi related to the lamina propria and with high definition were measured.

The parametric data were submitted to ANOVA with polynomial derivation (regression) with the GLM package of MINITAB 17.

Results and Discussion

The performance during the different phases was not related to the dosage of COS used (Table 4), and no episodes of diarrhea were observed.

The effect of the use of prebiotics for growth, feed intake, and feed efficiency shows a varied response and did not influence piglet performance during the nursery period, for prebiotics such as mannoooligosaccharides (CORASSA et al., 2012; SANTOS et al., 2010; SILVA et al., 2012), fructooligosaccharides (VISENTINI et al., 2008), and chitooligosaccharides (CHEN et al., 2009). However, with COS, some studies have reported better growth and feed efficiency in broilers (HUANG et al., 2005; LI et al., 2007) and piglets

(HAN et al., 2007; XU et al., 2013; WANG et al., 2012), with good results right after weaning. This pronounced effect for piglet growth seems to be related the ability of COS to act as a selective agent for gut bacteria, inhibiting the proliferation of noxious bacteria such as *E. coli* or *Bacillus cereus* (HAN et al., 2007; KUMAR et al., 2007; XIAO et al., 2014). This may be favorable for performance due to a reduction in the incidence of diarrhea (HAN et al., 2007; XU et al., 2013; WANG et al., 2012). In the present study, there was no significance for diarrhea because of the reduced sanitary challenge, reducing the benefits of COS.

Table 4. Zootechnical performance of piglets fed different chitosan (QOS) inclusion levels during the cumulative periods from 23 to 35, 50 and 63 days of age.

| Parameters | COS levels (ppm) | | | | Avg. | SE Mean | R ² * |
|----------------------|------------------|------|------|------|------|---------|------------------|
| | 0 | 50 | 100 | 150 | | | |
| 23 a 35 days | | | | | | | |
| Initial Weight (Kg) | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 | 0.25 | - |
| Final Weight (kg) | 10.6 | 10.6 | 10.7 | 10.8 | 10.7 | 0.29 | - |
| FI (g) | 458 | 474 | 454 | 502 | 472 | 10.40 | - |
| Growth (g) | 282 | 283 | 285 | 303 | 288 | 8.62 | - |
| FCR | 1.63 | 1.67 | 1.59 | 1.66 | 1.64 | 0.03 | - |
| 23 a 50 days | | | | | | | |
| Final Weight (kg) | 17.7 | 17.6 | 18.4 | 18.5 | 17.9 | 0.47 | - |
| FI (g) | 634 | 659 | 652 | 689 | 658 | 12.8 | - |
| Growth (g) | 411 | 410 | 436 | 438 | 424 | 9.00 | - |
| FCR | 1.54 | 1.61 | 1.50 | 1.57 | 1.55 | 0.02 | - |
| 23 a 63 days (total) | | | | | | | |
| Final Weight (kg) | 26.9 | 26.8 | 27.9 | 27.6 | 27.3 | 0.55 | - |
| FI (g) | 809 | 825 | 821 | 850 | 826 | 12.5 | - |
| Growth (g) | 510 | 508 | 532 | 524 | 518 | 9.89 | - |
| FCR | 1.59 | 1.62 | 1.54 | 1.62 | 1.6 | 0.02 | - |

R² of orthogonal polynomial regression for R² adjusted for a linear or quadratic effect.

For the relative weight of organs and the small intestine length (Table 5), a linear increase was observed for the relative weight of the large intestine and the dose of COS used. The use of

prebiotics has already been related to an increase in large intestine relative weight for monogastrics (PINHEIRO et al., 2006). This increase could be related to the modulatory effect of COS on the microbiota, leading to a higher prevalence of lactic acid bacteria that can ferment this oligosaccharide to produce short chain fatty acids (SCFA). The effects of these acids on the intestinal epithelia are related to an increase in cellular proliferation and activity, leading to an increase in the relative weight of this organ (ROBERFROID, 2002). Prebiotics and probiotics can change the fermentation profile and SCFA production and, even when compared

with antibiotics, promotes an increase in large intestine relative weight, related to benefits in performance and intestinal health (GARCÍA et al., 2014). Moreover, this increases the total number of bacteria in this organ and reduces the total amount of *E. coli* and *Clostridium spp.* (HAN et al., 2007). However, in this study, there was no quantification and differentiation of the gut flora, so, it was not possible to verify this effect of the studied product.

Table 5. Relative weight of the organs of the stomach, liver, spleen, small intestine and large, and small intestine length for the different levels of inclusion of chitoosoligosaccharide (COS).

| Parameters | COS levels (ppm) | | | | Avg. | SE Mean | R ² * |
|------------------------|------------------|------|------|------|------|---------|-------------------|
| | 0 | 50 | 100 | 150 | | | |
| <i>Relative Weight</i> | | | | | | | |
| Stomach (%) | 0.86 | 0.86 | 0.84 | 0.83 | 0.85 | 0.02 | - |
| Liver (%) | 2.3 | 2.4 | 2.1A | 2.30 | 2.27 | 0.01 | - |
| Spleen (%) | 0.22 | 0.25 | 0.22 | 0.26 | 0.23 | 0.04 | - |
| Small Intestine (%) | 5.50 | 5.30 | 5.46 | 5.10 | 5.34 | 0.11 | - |
| Large Intestine (%) | 2.14 | 2.24 | 2.48 | 2.58 | 2.36 | 0.08 | 0,97 ² |
| <i>Length</i> | | | | | | | |
| Small intestine (m) | 11.7 | 11.7 | 10.5 | 11.3 | 11.3 | 0.26 | - |

* R² of orthogonal polynomial regression for R² adjusted for a linear or quadratic effect.

¹ Values followed by letter A differed (P <0.05) from that observed in the control group.

² Equation: $y = 2.123 + 0.003152x$.

Another explanation for the high intestine higher relative weight is associated with the ability of prebiotics to change the cellular structure of

the intestine by promoting an increase in cellular density and in the total number of cells in the colon (HOWARD et al., 1995). The morphophysiological evaluation of different portions of the intestine (Table 6) indicated morphological changes in the jejunum and ileum with different levels of COS in the piglet feed. For the jejunum, higher levels of COS increased the villi high and the number of lesions linearly, while crypt depth was related to COS dosage in a quadratic way; in other words, an increase in crypt depth was observed at 50 and 100

ppm and with a reduction at 150 ppm (Figure 1). In the ileum, there was increasing of crypt depth with higher levels of COS until 150 ppm. Goblet cells were reduced with increasing COS levels. The lesion score and the high villi/crypt depth ratio were higher at 100 ppm COS and lower at 150 ppm in this segment.

Table 6. Morphology of the small intestine of piglets fed increasing levels of chitoooligosaccharide (COS).

| Parameters | COS levels (ppm) | | | | Avg. | SE Mean | R ² * |
|----------------------|------------------|-----|------|------|------|---------|------------------|
| | 0 | 50 | 100 | 150 | | | |
| <i>Duodenum</i> | | | | | | | |
| | 296 | 339 | 318 | 330 | 321 | 13,0 | - |
| Crypt (µm) | 260 | 239 | 272 | 246 | 254 | 7,22 | - |
| Globe cells (n) | 8,3 | 7,7 | 10,3 | 10,8 | 9,3 | 0,53 | - |
| Villi/Crypt relation | 1,1 | 1,3 | 1,1 | 1,3 | 1,2 | 0,07 | - |

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| <i>Jejunum</i> | | | | | | | |
|----------------------|------|------|-----|-----|-----|------|-------------------|
| Villi (µm) | 292 | 297 | 302 | 314 | 301 | 11,3 | 0,94 ¹ |
| Crypt (µm) | 212 | 238 | 241 | 215 | 226 | 7,30 | 0,99 ² |
| Globe cells (n) | 7,4 | 6,2 | 8,0 | 6,7 | 7,0 | 0,44 | - |
| Villi/Crypt relation | 1,5 | 1,5 | 1,3 | 1,5 | 1,5 | 0,07 | - |
| <i>Ileum</i> | | | | | | | |
| Villi (µm) | 269 | 303 | 322 | 289 | 296 | 10,0 | - |
| Crypt (µm) | 172 | 178 | 191 | 193 | 183 | 6,82 | 0,93 ³ |
| Globe cells (n) | 11,8 | 10,4 | 8,7 | 8,1 | 9,7 | 0,65 | 0,97 ⁴ |
| Villi/Crypt | 1,6 | 1,7 | 1,7 | 1,5 | 1,6 | 0,06 | 0,98 ⁵ |

Villi - Villi height; Crypt - Crypt depth.

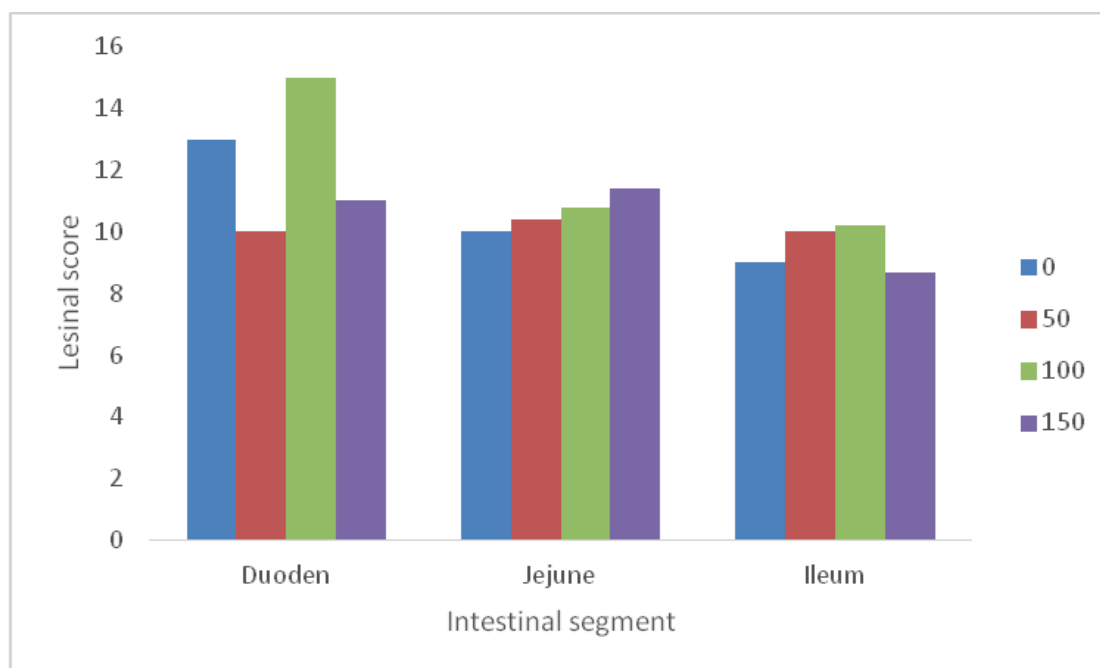
* R² of orthogonal polynomial regression for R² adjusted for a linear or quadratic effect.¹ Equation: $y = 283.5 + 7.1x$.² Equation: $y = 158.5 + 66.2x - 13x^2$.³ Equation: $y = 164.5 + 7.6x$.⁴ Equation: $y = 12.95 - 1.28x$.⁵ Equation: $y = 1.325 + 0.345x - 0.075x^2$.B - Equation: $y = 6.8 + 2.38x - 0.5x^2$. R² = 0.992.

Figure 1. Score of epithelial lesions in the duodenum, jejunum and ileum according to increasing levels of inclusion of COS in piglet feeding.

Source: Bracarense et al. (2012).

A - Equation: $y = 8.65 + 0.79x$. R² = 0.955.

The prebiotic effect on intestinal morphometry over the benefices bacteria selectivity in the gastrointestinal tract promotes positive modulation of intestinal morphometry and morphology due to an increase in SCFA production and a reduction in

toxic metabolites in the intestinal lumen, indicated by an increase in villi height and a better villi/crypt ratio in non-ruminant animals (GARCÍA et al., 2014; SANTANA et al., 2012; SILVA; NÖRNBERG, 2003), as seen with COS in piglets (HAN et al., 2007). In this study, the jejunum, which has excellent absorptive activity, demonstrated a direct correlation between villi height increasing levels of COS, corroborating the previous results of Liu et al. (2008) indicating that 200 ppm COS had a positive effect on this characteristic. Wang et al. (2012) obtained similar results working with 100 ppm nanoformulated COS, and Xu et al. (2013) obtained similar results with levels between 500 and 1000 ppm COS.

There was a quadratic effect in the ileum for the villi/crypt ratio, with a maximum response at 100 ppm COS in the piglet feed with a consequent reduction in this ratio with increasing levels. Similar results were found by Liu et al. (2008), working with 100 and 200 ppm COS in weaning piglets. Also, Xu et al. (2013) obtained a similar response for this intestinal segment, showing a higher villi/crypt ratio in the duodenum with 500 to 1000 ppm COS. Wang et al. (2012), however, did not observe any effect on intestinal morphology in piglets fed with 200, 400 or 600 ppm COS.

An increase in crypt depth in the jejunum and ileum was also observed in this study. These results are not frequent found with prebiotics in weaning piglets (UTIYAMA et al., 2004; BUDIÑO et al., 2005; LIU et al., 2008; TUCCI et al., 2011). However, shallow crypts are considered common with high levels of this prebiotic (500 to 1000 ppm) (XU et al., 2013) or when this prebiotic is associated with copper (WANG et al., 2012).

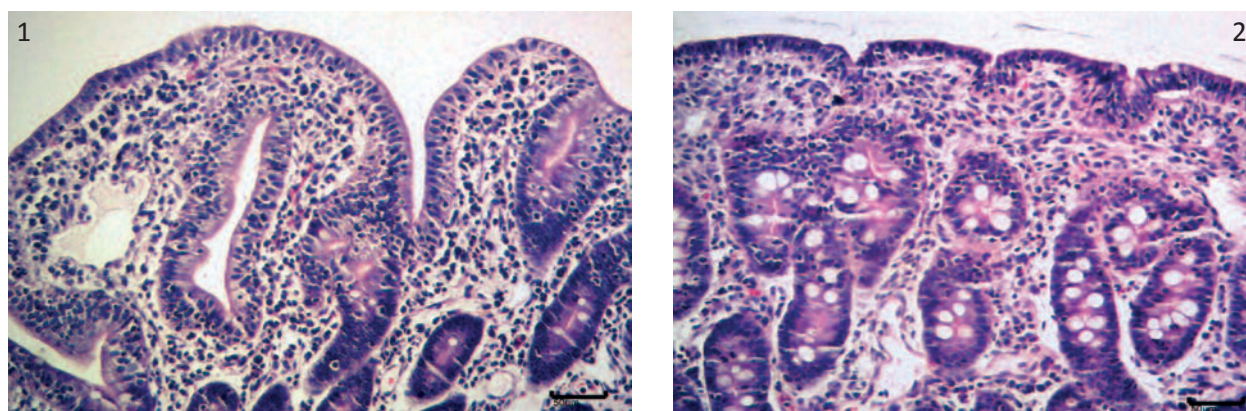
Crypt depth is a parameter that must be

considered in relation with villi height, because together provide a good evaluation of intestinal integrity. The ratio between villi height and crypt depth is indicative of the integrity of the epithelium via the process of cellular replication due to a mitosis-migration-extrusion that determines the digestive and absorptive ability of this tissue (PLUSKE et al., 1997). An increase in crypt depth corresponds, among other factors, with higher proliferative activity and to the antigenic features of the feed (HU et al., 2012).

Increasing crypt depth together with a reduction in the villi/crypt ratio indicates that cellular proliferation is happening to recover the villi apex (PLUSKE et al., 1997). However, the presence of COS in the intestinal lumen as a gut flora modulator could stimulate this proliferative activity due to SFCA production, which is a trophic agent in the intestinal mucosa. The trophic agents in this class include organic acids, and interact with the mucosa by accelerating mitotic activity and increasing the number of cells and, consequently, the size of the villus (BLAUT; CLAVEL, 2007; RICHARDS et al., 2005).

The highest incidence of lesions with the use of COS in the jejunum, characterized by a cubic epithelium, was found at 150 ppm; villi fusion was observed at 100 ppm COS. In the ileum, the highest incidence was also characterized by a cubic epithelium and villi fusion (Figures 1 and 2). The finding of a higher number of lesions in the ileum with the inclusion of 50 and 100 ppm COS could be related to the progressive reduction in goblet cells. The jejunum and the ileum are responsible for nutrient absorption (carbohydrates and amino acids), which is essential for piglet growth, and are

also related to the autonomous defense immune system of the intestine, i.e. mesenteric lymph nodes (mainly



jejunum) and Peyer's patches (ileum) (HERDT, 1999). A cubic epithelium and villi fusion interfere with the absorptive function of these segments and could lead to slower body growth in piglets.

Figure 2. Cubic epithelium with flattening of Villi (1) and fusion of Villi (2).

Goblet cells are responsible for mucin production, an integral part of intestinal self-defense against pathogens, enzymes, toxins, dehydration, and abrasions (VAN TASSELL; MILLER, 2011). Mucin is a glycoprotein with 80% carbohydrates and its constitution; thus it serves as a substrate for lactic acid bacteria, working as a prebiotic. A reduction in goblet cells could be associated, hypothetically, to the aggression stimulus reduction, to the selfish modulation of gut or to the fermentable oligosaccharide action to replace mucin by *Lactobacillus spp.*, leading to a reduction in the number of goblet cells (KIYOHARA et al., 2012).

The inclusion of COS (Table 7) affected the somatic cell count (SCC), indicated by an increase in lymphocytes in the duodenum and jejunum until the 100 ppm level, followed by a reduction with 150 ppm COS. In this way, the increasing in SCC in the duodenum with 100 ppm COS indicates cellular mobilization of the immune system, particularly for lymphocytes, and also an eosinophilic answer modulation that pointed a typical inflammatory

answer to the commensal stimulus of benefic bacteria for healthy status, characterized by the development of tolerance in the intestinal epithelium. The activation capacity of the immune system by prebiotics is related to a modulatory effect on the gut flora (HOLSCHER et al., 2012) and to stimulation of the immune system via different mechanisms of action (SILVA; NÖRNBERG, 2003).

The antigenic stimulus of prebiotics and gut modification promotes, via epithelial growth factor receptors (EGF-R), an inflammatory cell response that increases epithelial barrier function, modulates mucus secretion and IgA production via cytokines, and stimulates antimicrobial peptide production and intraepithelial lymphocytes production; this culminates in macrophage activation (SILVA; NÖRNBERG, 2003). Prebiotics can affect these processes directly or indirectly via beneficial bacteria, particularly *Lactobacillus*, which are able to induce tolerance and suppress the inflammatory cascade in the absence of pathogenic microorganisms. This has positive effects on piglet intestinal morphometry, permits tolerance to commensal bacteria, and activates the immune defense against pathogenic agents, but due to an effectiveness activation (HARDY et al., 2013).

Recently, Xiao et al. (2014) identified a mechanism of immune regulation related to the use of 300 ppm COS in weaning piglet feed associated

with TLR4 protein expression, which is a key protein for commensal bacteria recognition in the intestine that is overexpressed during the inflammatory process in response to IL-1 β and IL-6 in the jejunal mucosa.

Table 7. Somatic cell counts (SCC) and their differentiations in the small intestine of piglets fed with increasing levels of chitooligosaccharide (COS).

| Parameters | COS levels (ppm) | | | | Avg. | SE Mean | R ² * |
|-----------------|------------------|------|--------|--------|------|---------|-------------------|
| | 0 | 50 | 100 | 150 | | | |
| Duodenum | | | | | | | |
| SCC (n) | 72.6 | 71.3 | 81.7 A | 72.7 | 74.6 | 6.84 | - |
| Lymphocytes (n) | 21.2 | 30.1 | 29.6 | 23.6 | 26.1 | 2.56 | 0.99 ² |
| Plasmocytes (n) | 37.6 | 32.6 | 42.8 | 33.2 | 36.5 | 2.67 | - |
| Eosinophils (n) | 13.9 | 8.7 | 9.3 | 15.9 | 11.9 | 1.60 | 0.99 ³ |
| Jejunum | | | | | | | |
| SCC (n) | 49.7 | 61.2 | 56.6 | 65.6 | 58.3 | 5.31 | - |
| Lymphocytes (n) | 19.1 | 25.7 | 24.9 | 24.6 | 23.6 | 1.56 | - |
| Plasmocytes (n) | 14.6 | 23.9 | 17.0 | 18.5 | 18.5 | 1.88 | - |
| Eosinophils (n) | 15.9 | 11.6 | 14.7 | 22.6 | 16.2 | 1.88 | 0.98 ⁴ |
| Ileum | | | | | | | |
| SCC (n) | 68.3 | 71.8 | 68.7 | 88.6 | 74.4 | 6.88 | - |
| Lymphocytes (n) | 20.2 | 24.9 | 22.8 | 28.7 | 24.2 | 2.47 | - |
| Plasmocytes (n) | 7.8 | 9.2 | 7.4 | 19.7 A | 11.0 | 1.61 | - |
| Eosinophils (n) | 40.3 | 37.7 | 38.5 | 40.2 | 39.2 | 2.60 | - |

The number of lymphocytes, plasma cells and eosinophils were counted in the lamina propria region, in three random fields per sample, using the 40x objective.

* R² of orthogonal polynomial regression for R² adjusted for a linear or quadratic effect.

² Equation: $y = 5.825 + 19.295x - 3.725x^2$.

³ Equation: $y = 25.05 - 14.09x + 2.95x^2$.

⁴ Equation: $y = 25.65 - 12.93x + 3.05x^2$.

The improved performance of piglets fed with COS is generally associated with nutrient digestibility, gut flora modulation and to reduced diarrhea incidence and an improvement in the feces score (HAN et al., 2007; XU et al., 2013; WALSH et al., 2013). However, enteric challenge was not performed in this experiment, which could be the main reason for the similar performance at all doses of COS. Therefore, besides the higher large intestine weight, villi and cell proliferation tax increasing, and tissue immune system activation,

the energy expenditure did not interfere with animal performance regarding growth and the feed conversion ratio.

Conclusions

The increasing levels of COS in piglet feed led to better intestinal morphophysiological and stimulated tissue immunity, but did not influence the productive performance of the animals, probably because the animals were not submitted to adverse enteric conditions. The level of 100 ppm was the best regarding morphometry and intestinal organs, avoiding the presence of epithelial lesions observed in the jejunum with increasing levels of this prebiotic.

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