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New findings of intestinal alkaline phosphatase: effects on intestinal and organ health of piglets challenged with ETEC F4 (K88)

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ABSTRACT - The aim of this study was to assess the addition of intestinal alkaline phosphatase (IAP) to diets on the count of bacterial populations, pH of digestive organ contents, histopathological description, proinflammatory markers, hepatic glycogen reserve, and diarrhoea incidence of piglets challenged with *Escherichia coli*. Sixty-four crossbred piglets (7.16±0.28 kg body weight, 25-days-old) were assigned to four treatments in a completely randomised block design: negative control (NC), NC + antibiotic (ANT), NC + 15 mg IAP, or NC + 30 mg IAP kg^{-1} of diet, eight replications of two piglets per experimental unit. All piglets were orally challenged with 6 mL of a solution containing enterotoxigenic *Escherichia coli* K88 at 10^6 CFU mL^{-1} at 15 days of experimentation. The study lasted for 19 days. At the end of the experimental period, the piglets were slaughtered (six animals per treatment). Enterobacteriaceae in caecum and colon was lower in piglets on 30 mg IAP than with ANT and NC, ANT or 15 mg IAP, respectively. Enterobacteriaceae adhered to the mesenteric lymph nodes (MLN) was greater in piglets fed ANT than the other treatments. Lactic acid bacteria (LAB) count in caecum was greater in piglets fed NC and ANT. In MLN, LAB count was greater in ANT and 30 mg IAP-fed piglets compared with 15 mg IAP. Piglets in 30 mg IAP in diet showed a tendency for lowering tissue necrosis compared with NC or ANT. Piglets fed 30 mg IAP showed a reduction in diarrhoea incidence in the pre- and post-challenge compared with 15 mg IAP and all other treatments, respectively. Based on the criteria, addition of 30 mg IAP to diet inhibits Enterobacteriaceae population and suggests a potential effect in mitigating intestinal injuries, as observed in piglets in the NC for some of the parameters investigated.

Keywords: alkaline phosphatase, bacterial populations, diarrhoea incidence, intestinal histopathology, proinflammatory marker, weanling pigs

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1. Introduction

Effective functionality of the gastrointestinal tract (GIT) and intestinal health are important factors in the piglet nutrition (Celi et al., 2017). The health of GIT has attracted a lot of attention (Pluske et al., 2018), and aspects related to intestinal microbiota, immune system, management practices, and nutritional alternatives are critical factors in the overall health (Liu et al., 2018).

As auxiliary and complementary mechanisms to mitigate these effects mentioned above, piglets have enteroendocrine cells that play important roles, such as pathogen detection, synthesis and release of neuropeptides (Moeser et al., 2017), recognition of pathogenic signalling molecules, and interleukin secretion (Pluske et al., 2018). Furthermore, intestinal microbiota plays a role in the synthesis of beneficial nutrients, and on the deleterious effects of inflammation (Celi et al., 2017).

However, pathogenic infection is one of the challenges that affect piglets (Sun and Kim, 2017). Any discussion in this critical post-weaning period highlights the potential impacts of enterotoxigenic *Escherichia coli* (ETEC) strains (Yi et al., 2016), including F4 $(K88)^+$ (Pan et al., 2017), because *E. coli* diarrhoea can cause piglet mortality (Gresse et al., 2017).

In this way, the attention of the swine industry and measures related to intestinal health have increased considerably, due to changes that reduce the use of performance-enhancing antibiotics. In this sense, a wide range of products such as feed additives that aim to improve GIT health have been investigated (Jayaraman and Nyachoti, 2017).

Studies have reported the use of intestinal alkaline phosphatase (IAP) isoenzyme, produced by intestinal epithelial cells to play a role in maintaining intestinal health (Lallès, 2014), due to decreased inflammation in the colon (Alam et al., 2014), reduction of inflammatory activity of tumour necrosis factor alpha (TNF-α) (Moss et al., 2013), marked reduction of markers expression as the neutrophil markers calgranulin A, lipocalin 2, and interleukin-1β (Martínez-Moya et al., 2012), ability to promote bacterial growth (Malo et al., 2014), and modulation of intestinal pH (Brun et al., 2014).

Therefore, the aim of this study was to assess the addition of IAP in diets on intestinal health by counting of bacterial populations in the intestinal contents and adhered to mesenteric lymph nodes, pH of digestive organ contents, histopathological description of the jejunum, immunohistochemistry of proinflammatory markers in the liver and jejunum, hepatic glycogen reserve, and diarrhoea incidence of piglets challenged with *E. coli* F4 (K88).

2. Material and Methods

The study was conducted on an experimental farm located in Marechal Cândido Rondon, Paraná, Brazil (24°31'52" S and 54°01'03" W). Piglets were carefully managed to avoid unnecessary discomfort, and all experimental procedures were approved by the local Research Ethics Committee (Protocol No. 16/19).

2.1. Experimental design, animals, housing, and diets

A total of 64 crossbred piglets (Landrace × Large White, Agroceres♂ and DanBred♀), entire males weaned at 25-days-old with an initial body weight of 7.16±0.28 kg were allocated in a completely randomised block design consisting of four treatments and eight replications, totalling 32 experimental units, with two animals per experimental unit.

The animals were weighed (Rinnert digital scale, model BPW-5000; Braço do Trombudo, SC, Brazil), identified with numbered ear tags, and housed in a nursery facility at the beginning of the experimental period. The suspended pens (1.50 × 1.03 m) were made of polyethylene plastic flooring, equipped with nipple drinking fountains and gutter feeders, arranged in two rows and divided by a central corridor, where the piglets remained for a period of 19 days.

The ambient temperature and relative humidity were recorded using a data logger with digital display (UNI-T UT 330B digital USB; Beijing, China), which was installed in the centre of the experimental building. The minimum recorded temperature of the internal environment was 19.1±5.2 °C, and the maximum was 29.7±5.5 °C. The nursery facility was ventilated with fans, exhaust fan, and tilting-type windows. The heating of the experimental pens was controlled using individual infrared incandescent lamps.

The diets were formulated to meet the piglets' requirements for pre-starter I and II phases, following the nutritional recommendations proposed by Rostagno et al. (2017). The experimental treatments (Table 1) were composed of a negative control (NC, diet without feed additive), NC + antibiotic (ANT,

Table 1 - Centesimal composition and chemical and calculated values of experimental diets provided to piglets in the experimental period (%, as-fed basis)

Item	Negative control (NC)[1]		NC + ANT		NC + 15 mg of IAP		NC + 30 mg of IAP	
	PI[2]	PII	PI	PII	PI	PII	PI	PII
Grain corn 7.86% CP	40.10	50.75	40.07	50.72	35.99	46.64	31.88	42.53
Soybean meal 45.4% CP	19.75	17.84	19.75	17.84	20.48	18.57	21.22	19.31
Whey powder 12.3% CP	14.66	9.33	14.66	9.33	14.66	9.33	14.66	9.33
Extruded semi-whole soy[3]	12.00	10.00	12.00	10.00	12.00	10.00	12.00	10.00
Sugar	5.00	4.00	5.00	4.00	5.00	4.00	5.00	4.00
Fish meal 53.0% CP	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Soybean oil	1.96	1.61	1.97	1.62	3.35	3.00	4.73	4.39
Dicalcium phosphate	1.39	1.33	1.39	1.33	1.39	1.34	1.40	1.34
Limestone	0.89	0.80	0.89	0.80	0.88	0.79	0.87	0.78
L-lysine HCl 78%	0.40	0.42	0.40	0.42	0.39	0.41	0.38	0.39
L-threonine 96.8%	0.26	0.26	0.26	0.26	0.26	0.25	0.26	0.25
DL-methionine 99.5%	0.24	0.21	0.24	0.21	0.24	0.22	0.24	0.22
L-tryptophan 99%	0.04	0.05	0.04	0.05	0.04	0.04	0.04	0.04
Common salt	0.19	0.29	0.19	0.29	0.19	0.29	0.19	0.29
Mineral premix[4]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Vitamin premix[4]	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
ANT[5]	-	-	0.015	0.015	-	-	-	-
Microencapsulated IAP[6]	-	-	-	-	2.00	2.00	4.00	4.00
Total (%)	100	100	100	100	100	100	100	100
Calculated values								
Crude protein (%)	21.42	19.87	21.42	19.87	21.42	19.87	21.42	19.87
Metabolisable energy (kcal kg^{-1})	3,400	3,375	3,400	3,375	3,400	3,375	3,400	3,375
Total calcium (%)	1.068	0.973	1.068	0.973	1.068	0.973	1.068	0.973
Available phosphorus (%)	0.528	0.481	0.528	0.481	0.528	0.481	0.528	0.481
Sodium (%)	0.224	0.219	0.224	0.219	0.224	0.219	0.224	0.219
Digestible lysine (%)	1.451	1.346	1.451	1.346	1.451	1.346	1.451	1.346
Digestible methionine + cysteine (%)	0.813	0.754	0.813	0.754	0.813	0.754	0.813	0.754
Digestible threonine (%)	0.972	0.902	0.972	0.902	0.972	0.902	0.972	0.902
Digestible tryptophan (%)	0.276	0.256	0.276	0.256	0.276	0.256	0.276	0.256
Lactose (%)	11.00	7.00	11.00	7.00	11.00	7.00	11.00	7.00
Analysed values (%)								
Crude protein	21.49	19.88	21.40	19.81	21.41	19.85	21.47	19.86
Dry matter	93.60	91.85	93.52	92.40	93.49	92.39	93.49	92.62
Organic matter	87.18	86.01	87.00	86.49	87.26	86.43	87.26	86.82
Mineral matter	6.34	5.92	6.53	5.91	6.22	6.00	6.22	5.80
Neutral detergent fibre	10.42	11.22	10.55	11.43	9.23	10.84	9.10	10.74
Acid detergent fibre	2.43	2.26	2.50	2.22	2.32	2.14	2.29	2.09
Total calcium	1.05	1.02	1.05	1.02	1.04	1.02	1.04	1.02
Total phosphorus	0.80	0.70	0.81	0.72	0.76	0.75	0.76	0.75
Total magnesium	0.11	0.10	0.12	0.10	0.11	0.10	0.11	0.11
Ether extract	2.43	4.25	2.41	4.31	4.17	6.38	8.28	7.58
Gross energy (kcal kg^{-1})	4,012	3,906	3,945	3,851	4,164	4,084	4,295	4,253

[1] Experimental treatments = NC: negative control diet without feed additive; ANT: antibiotic.
[2] Experimental phases - PI and PII: pre-starter I and II.
[3] Extruded semi-whole soybean 43.16%.
[4] Nutritional levels per kg of premix: Mn sulfate, 120 mg kg^{-1}; Zn oxide, 160 mg kg^{-1}, Fe sulfate, 120 mg kg^{-1}; Cu sulfate, 20 mg kg^{-1}; I, 2 mg kg^{-1}; sodium selenite, 1.2 mg kg^{-1}; vitamin K3, 12,800 mg kg^{-1}; vitamin B1, 6,400 mg kg^{-1}; vitamin B2, 16,000 mg kg^{-1}; vitamin B6, 6,400 mg kg^{-1}; niacin, 98,260 mg kg^{-1}; pantothenic acid, 32,340 mg kg^{-1}; folic acid, 1,920 mg kg^{-1}; vitamin B12, 64,000 mcg kg^{-1}; biotin, 640,000 mcg kg^{-1}; vitamin A, 32,000 KIU kg^{-1}; vitamin D3, 6,400 KIU kg^{-1}; vitamin E, 80,000 IU kg^{-1}.
[5] Tiamulin: 150 mg kg^{-1} diet.
[6] Intestinal alkaline phosphatase type I-S obtained from bovine intestinal mucosa (Sigma-Aldrich Corporation).

150 mg tiamulin kg^{-1} diet as active ingredient) as positive control, NC + 15 mg IAP (P7640, type I-S obtained from bovine intestinal mucosa, lyophilised powder, ≥10 DEA units mg^{-1} solid which hydrolyses 1.0 micromole of P-nitrophenyl phosphate per min at pH 9.8 at 37 °C; Sigma-Aldrich Corporation) kg^{-1} diet or NC + 30 mg IAP kg^{-1} diet. The experimental diets were given in mash form, with minimal differences in the amount of corn and soybean meal for the addition of IAP.

2.2. Processing of IAP microencapsulation

The microencapsulation process consisted of the dilution of the enzyme in rice starch (RS), propylene glycol ester (PGE) + palmitic acid (PA) in the proportion of 1 g 1.333 kg^{-1}. The final composition of the vehicle used in the microencapsulation process presented the following proportions: 50% RS, 30% PGE + 20% PA.

2.3. Bacterial strain and challenge procedure

At 15 days of experimentation (day of infection), piglets were subjected to an 8-h fasting and were challenged individually, receiving 6 mL of bacterial suspension containing a dose of 10^6 CFU mL^{-1} of an ETEC F4 strain (K88), isolated from weanling pigs' faeces (post-weaned with 21-days-old). A single colony from each plate was collected and spread onto brain heart infusion medium agar and incubated at 36±1 °C for 24 h until reaching the concentration of 1.0×10^9 CFU mL^{-1}. Subsequently, serial dilutions were performed in saline solution (0.9% NaCl) to reach the concentration of 1.0×10^6 CFU mL^{-1}. Inoculation of animals was carried out using a syringe (10 mL) without needle and slowly dripping the ETEC inoculum into each piglet's throat to trigger the swallowing reflex and minimise the inoculum passage into the lungs.

2.4. Slaughtering and sampling

At 19 days of experimentation, six animals were fasted for six hours, stunned by electronarcosis (240 volts for 3 s) and euthanised by exsanguination to collect data and biological samples for bacterial populations count in the contents of jejunum, caecum, colon, and adhered lymph nodes, measuring the pH of the digestive tract contents and histopathological analysis of the jejunum. For the expression analysis of factors related to inflammation, segments of the jejunum and liver were collected for immunohistochemical evaluation. The choice of animal to be slaughtered was according to the closest body weight of the average of treatment in different pens.

Samples of intestinal content from jejunum, caecum, and colon were collected, as well as the mesenteric lymph nodes (MLN), which were subjected to Enterobacteriaceae and lactic acid bacteria (LAB) counts. The samples were packed in sterile plastic containers, identified, and transported under refrigeration (4 °C) for laboratory analysis. Mesenteric lymph nodes were aseptically macerated with the aid of a sterile mortar and pestle. Subsequently, one gram of the faecal content of the digestive tract and macerated MLN was transferred to identified sterile tubes and subjected to serial dilution in saline solution (0.9% NaCl). The dilution 10^{-1} (1 g of sample with 9 mL of saline solution) was homogenised in vortex (Phoenix brand, AP 56 model; Araraquara, SP, Brazil) for 30 s. The remaining dilutions, up to 10^{-6} (caecum and colon) and 10^{-5} (mesenteric lymph nodes), were homogenised in vortex (Phoenix brand, AP 56 model; Araraquara, SP, Brazil) for 10 s. An aliquot of 100 µL of each dilution was spread onto EMB levine agar (Kasvi, São José do Pinhais, PR, Brazil), and plates were incubated in aerobic greenhouses (Eletrolab brand, EL 202 model; Curitiba, PR, Brazil) overnight at 37 °C for 24 h for the Enterobacteriaceae count. For the LAB count, an aliquot of 1 mL of each was inoculated into MRS agar (Acumedia - Prolab, São Paulo, SP, Brazil), using the pour plate method and incubated in anaerobic greenhouses (De Leo brand, single model; Porto Alegre, RS, Brazil) at 37 °C for 44 h under restricted oxygen conditions, according to the methodology described by Weedman et al. (2011).

Measurement of the pH of the stomach, jejunum, caecum, and colon contents was performed using a digital pH metre (Hanna Instruments Inc., Rhodes Island, USA, model HI 99163; Smithfield, RI, United States of America) through the insertion of a unipolar electrode, adopting the methods described by Manzanilla et al. (2004). Access to the contents present in the stomach was made with an incision in the oesophageal region (approximately 2 cm from the oesophagus), and the pH was measured after homogenisation of the digestive contents. After digesta homogenisation in the intestine, the pH was measured in the median part of the jejunum (150 cm from the ileocecal junction) and in the caudal parts of the caecum and colon (Guo et al., 2001).

Segments of approximately 3 cm in length from the jejunum (extracted at 150 cm from the ileocecal junction) (Guo et al., 2001) and liver fragments were collected, washed with saline solution (0.9% NaCl), and stored in sterile plastic pots with a volume of 50 mL containing 10% buffered formaldehyde solution (37.5% commercial formaldehyde, distilled water, mono and dibasic sodium phosphate) for 48 h, then transferred and kept in a 70% alcohol solution.

Subsequently, the samples were sent to the laboratory (Cascavel, PR, Brazil) where they were paraffin-embedded and microtomised for slides mounting. The paraffin blocks containing the samples were cut in a microtome (Leica RM2245, Leica Biosystems, São Paulo, Brazil), and sections were performed and transferred to the slides. The slides were stained with haematoxylin and eosin for histopathological description (Gao et al., 2000) and periodic acid-Schiff staining for hepatic glycogen reserve (HGR) (Tuin et al., 2006). The parameters analysed in the histopathological description of the jejunum epithelium were the presence of infiltrate, hyperaemia, desquamation, coccidiosis, lumps, rods, cysts, mucus, goblet cells, and necrosis. The stained slides were viewed and photographed using an AxioCam MRC camera (Carl Zeiss®, Göttingen, Germany) coupled to the Axio Scope A1 microscope (Carl Zeiss®, Jena, Germany). The Axionvision Se 64 software (Carl Zeiss®, Thornwood, USA) was used for the analyses described above.

For proinflammatory reactivity by immunohistochemical evaluation (TNF-α, cyclooxygenase-2, toll-like receptor 4 and proliferating cell nuclear antigen), the same samples used for histopathological analysis were used to create the blocks by the tissue microarray technique, described by Engracia Filho et al. (2017). Cyclooxygenase-2 (COX-2) immune expression was assessed using the polyclonal anti-Cox-2 antibody (Dako, Glostrup, Denmark). The percentage area immunolabeled with COX-2 (μm^2) was calculated by evaluating seven images for each replicate, according to the methodology of Álvares et al. (2018). Afterwards, the slides were scanned on the Axio Scan.Z1 scanner (Carl Zeiss®, Jena, Germany) and analysed using Image Pro Plus 4 software (Media Cybernetics Inc., Rockville, USA).

To identify TNF-α in the fragments (μm^2), the anti-TNF-α primary antibody (ABCam, Cambridge, UK) was used in the preparation of the slides. Tumour necrosis factor alpha positive cells were counted using images obtained from the Olympus BX40 microscope with a 40X objective lens. Five random fields of the jejunum and liver were photographed in each replicate, and subsequently the average immunolabeled cells count was obtained. Immunohistochemical analysis of toll-like receptor 4 (TLR4, in %) was made using a TLR4/CD284 polyclonal antibody (Product PA-23125, ThermoFisher Scientific) at 1:100 dilution. Immunohistochemical analysis of proliferating cell nuclear antigen (PCNA, in %) was performed using anti-PCNA polyclonal antibody (Product PA5-32541) at 1:100 dilution.

2.5. Diarrhoea incidence

The diarrhoea incidence was recorded daily for each pen, in the morning at 9:00 h, priori to cleaning the experimental unit. Presence or absence of diarrhoea (liquid faeces on the floor and/or dirty anal region) was calculated as the proportion of animals with diarrhoea in each phase during the experiment. Diarrhoea incidence (%) = [(n of piglets with diarrhoea) ÷ (n of piglets × n of days per experimental phase)] × 100, in which the number (n) of piglets with diarrhoea was the sum of the number of piglets (16 piglets/treatment) with diarrhoea every day in each phase (Huang et al., 2004).

2.6. Statistical analyses

Before evaluating the result of analysis of variance (ANOVA), the standardised residuals analysis of Student was performed to identify outliers (values greater than or equal to three standard deviations). The normality of experimental errors and the homogeneity of error variances among treatments for the several variables were previously evaluated using the Shapiro-Wilk and Levene tests, respectively. A total of eight replications were considered for the diarrhoea incidence analysis, and six replications were euthanised and analysed for the other variables. For the characteristics analysed, the statistical model used was:

$$Y_{ijk} = \mu + T_i + b_j + \varepsilon_{ijk},$$

in which Y_{ijk} = average observation of the dependent variable in each plot, measured in the *i*-th treatment class, at the *j*-th block, and in the *k*-th replication; μ = effect of the overall average; T_i = fixed effect of treatment classes, for *i* = (1, 2, 3, and 4); b_j = block effect, for *j* = (1 and 2); ε_{ijk} = random error of the plot associated with *i*-th level, *j*-th block, and *k*-th replication. For the counting characteristic of bacterial populations, the data were transformed into logarithm (base 10). The effects of the experimental treatment classes on the dependent variables were verified through ANOVA. Comparisons between treatment averages were performed according to Tukey's post-hoc test at 5% probability.

For statistical analysis of histopathological description, the generalised linear model was fitted in each distribution and binding function. For the diarrhoea incidence, the data were transformed into binary values, using the binomial distribution, wherein: 0 = diarrhoea absence and 1 = diarrhoea presence, and presented as percentage results. Generalised linear model used was represented by the systematic portion:

$$\eta = \mu + T_i + b_j,$$

wherein μ was the effect associated with the overall average, T_i was the effect associated with *i*-th treatment class, for *i* = (1, 2, 3, and 4), and b_j was the effect associated with *j*-th block, for *j* = (1 and 2). The significance of the coefficients associated with the effect of experimental diets was verified with the type III analysis. The criterion to evaluate the fit quality of the model was verified by the Akaike information criterion. Average comparisons were performed using a test of the difference between the lsmeans, through the χ^2 statistic. All statistical analyses were performed using the procedures of the statistical software SAS (Statistical Analysis System, University Edition). Data were presented as means with standard error of the mean.

3. Results

3.1. Counts of intestinal and mesenteric lymph node-adhered microbial populations and pH of digestive tract contents

Enterobacteriaceae count in caecum content was lower (P = 0.002) in piglets fed 30 mg IAP compared with those fed ANT and 15 mg IAP (Table 2). In colon, Enterobacteriaceae count was also lower in piglets fed 30 mg IAP than in those fed NC, ANT, or 15 mg IAP (P = 0.007). Enterobacteriaceae population adhered to MLN was greater (P = 0.006) in piglets fed ANT compared with the other treatments. Piglets fed NC or ANT showed the highest (P<0.01) LAB count in the caecum content. In MLN, there was a treatment effect (P = 0.013) on LAB count, for which ANT- and 30 mg IAP-fed piglets showed greater count than 15 mg IAP-fed piglets (Table 2).

There was no treatment effect (P>0.05) on pH of the digestive tract contents (Table 3).

Table 2 - Effect of intestinal alkaline phosphatase on the Enterobacteriaceae and lactic acid bacteria counts (Log_{10} CFU g^{-1}) of piglets at 44-days-old challenged with *Escherichia coli* F4 (K88)

Item	Experimental treatment[1]				SEM	P-value
	NC	ANT	15 IAP	30 IAP		
	Enterobacteriaceae counts (Log_{10} CFU g^{-1})					
Caecum	7.34bc	8.06a	7.77ab	7.02c	0.130	0.002
Colon	7.22a	7.56a	7.04a	6.18b	0.140	0.007
Mesenteric lymph nodes	4.21b	5.04a	4.47b	4.50b	0.116	0.006
	Lactic acid bacteria counts (Log_{10} CFU g^{-1})					
Caecum	9.22a	9.17a	8.73b	8.33c	0.060	0.000
Colon	8.45	8.81	8.82	8.76	0.130	0.240
Mesenteric lymph nodes	6.15ab	6.24a	5.64b	6.30a	0.116	0.013

SEM - standard error of the mean.
[1] Experimental diets = NC: negative control diet without feed additive; ANT: diet with the addition of antibiotic; 15 IAP: control diet + 15 mg intestinal alkaline phosphatase kg^{-1} of diet; 30 IAP: control diet + 30 mg intestinal alkaline phosphatase kg^{-1} of diet.
Averages followed by different lowercase letters in row differ according to Tukey's test at 5% probability.

Table 3 - Effect of intestinal alkaline phosphatase on the pH of digestive tract contents of piglets at 44-days-old challenged with *Escherichia coli* F4 (K88)

Item	Experimental treatment[1]				SEM	P-value
	NC	ANT	15 IAP	30 IAP		
	pH of digestive tract contents					
Stomach	3.70	3.06	3.36	3.53	0.133	0.370
Jejunum	5.60	5.90	5.98	5.53	0.121	0.476
Caecum	5.37	5.33	5.36	5.45	0.042	0.597
Colon	5.61	5.79	5.64	5.82	0.047	0.289

SEM - standard error of the mean.
[1] Experimental diets = NC: negative control diet without feed additive; ANT: diet with the addition of antibiotic; 15 IAP: control diet + 15 mg intestinal alkaline phosphatase kg^{-1} of diet; 30 IAP: control diet + 30 mg intestinal alkaline phosphatase kg^{-1} of diet.

3.2. Histopathological description of the piglet jejunum

There was no effect of experimental treatment on the cell infiltrate (P = 0.200), epithelial desquamation (P = 0.174), bacterial lumps (P = 0.130), mucus (P = 0.428), goblet cells (P = 0.400), and epithelial hyperaemia (P = 0.101) of piglets challenged with ETEC F4 (Table 4). However, the addition of 30 mg IAP in diet showed a tendency (P = 0.086) for lowering tissue necrosis in piglets (Table 4).

Table 4 - Effect of intestinal alkaline phosphatase on the histopathological description of the jejunum of piglets at 44-days-old challenged with *Escherichia coli* F4 (K88)

Item[1]	Experimental treatment[2]				P-value
	NC	ANT	15 IAP	30 IAP	
Cell infiltrate	1.16	0.41	0.66	0.41	0.200
Epithelial hyperaemia	1.41	1.16	1.41	1.08	0.101
Epithelial desquamation	1.58	0.83	1.16	1.16	0.174
Coccidiosis	0	0	0	0	-
Bacterial lumps	0.66	0	0.16	0	0.130
Rods	0	0	0	0	-
Cysts	0	0	0	0	-
Mucus	0.16	0	0.16	0	0.428
Goblet cells	1.91	2.16	2.25	2.00	0.400
Tissue necrosis	0.25	0.50	0.16	0.08	0.086

[1] Histopathological description: 0 = absent; 1 = discrete; 2 = moderate; 3 = intense.
[2] Experimental diets = NC: negative control diet without feed additive; ANT: diet with the addition of antibiotic; 15 IAP: control diet + 15 mg intestinal alkaline phosphatase kg^{-1} of diet; 30 IAP: control diet + 30 mg intestinal alkaline phosphatase kg^{-1} of diet.

3.3. Proinflammatory markers on liver and jejunum epithelium and hepatic glycogen reserve

There was no effect of the treatments on the TNF-α concentration in the jejunum (P = 0.454) and liver (P = 0.217), COX-2 activity in the jejunum (P = 0.285) and liver (P = 0.624), TLR4 concentration in the jejunum (P = 0.319) and liver (P = 0.243), PCNA in the jejunum (P = 0.668) and liver (P = 0.127), nor on HGR (P = 0.236), although piglets in NC showed a slight decrease (19.16%) in the HGR compared with those that consumed 30 mg IAP (Table 5).

Table 5 - Effect of intestinal alkaline phosphatase on the tumour necrosis factor alpha (TNF-α), cyclooxygenase 2 (COX-2), Toll-like receptor 4 (TLR4) activity, and proliferating cell nuclear antigen (PCNA) in the jejunum and liver, and hepatic glycogen reserve of piglets at 44-days-old challenged with *Escherichia coli* F4 (K88)

Item	Experimental treatment[1]				SEM	P-value
	NC	ANT	15 IAP	30 IAP		
			Jejunum			
TNF-α (μm²)	0.25	0.18	0.08	0.06	0.044	0.454
COX-2 (μm²)	12.59	15.71	6.70	14.70	1.657	0.285
TLR4 (%)	12.33	14.20	15.55	10.61	1.165	0.319
PCNA (%)	10.46	7.72	8.80	9.38	0.811	0.668
			Liver			
TNF-α (μm²)	3.48	4.89	4.08	2.57	0.381	0.217
COX-2 (μm²)	16.97	14.66	11.31	17.57	1.639	0.624
TLR4 (%)	19.70	15.59	11.04	20.78	2.016	0.243
PCNA (%)	8.73	15.36	15.50	14.90	1.211	0.127
Glycogen reserve (%)	18.49	31.20	37.65	29.31	3.251	0.236

SEM - standard error of the mean.
[1] Experimental diet = NC: negative control diet without feed additive; ANT: diet with the addition of antibiotic; 15 IAP: control diet + 15 mg intestinal alkaline phosphatase kg^{-1} of diet; 30 IAP: control diet + 30 mg intestinal alkaline phosphatase kg^{-1} of diet.

3.4. Diarrhoea incidence

The ability of IAP in piglet diets to reduce the diarrhoea incidence was verified pre-challenge and post-challenge. In pre-starter I phase, the average DI reduction presented by piglets fed 30 mg of IAP was 13.85% when compared with those that received 15 mg IAP. For the pre-starter II phase, there was a difference (P = 0.044) of the treatments, in which the piglets that consumed the diet containing 30 mg of IAP showed a 24% reduction in diarrhoea incidence compared with the 15 mg IAP treatment (Table 6). The main effect (P = 0.009) of diarrhoea incidence reduction was with the addition of 30 mg

Table 6 - Effect of intestinal alkaline phosphatase on diarrhoea incidence (DI) in piglets challenged with *Escherichia coli* F4 (K88)

Item[1]	Experimental treatment[2]				SEM	P-value
	NC	ANT	15 IAP	30 IAP		
	Pre-starter I phase (7.16 to 8.89 kg) – 25 to 35 days of age					
DI (%)	23.08	26.15	32.31	18.46	4.003	0.201
	Pre-starter II phase (8.89 to 11.19 kg) – 35 to 44 days of age					
DI (%)	20.00ab	28.00ab	38.00a	14.00b	3.454	0.044
	Post-challenge phase (9.42 to 11.19 kg) – 40 to 44 days of age					
DI (%)	33.33a	16.66b	44.44a	5.56c	4.754	0.009
	Total period (7.16 to 11.19 kg) – 25 to 44 days of age					
DI (%)	23.31ab	25.56ab	36.09a	15.04b	2.987	0.007

SEM - standard error of the mean.
[1] Diarrhoea incidence was calculated as the proportion of animals with diarrhoea.
[2] Experimental diets = NC: negative control diet without feed additive; ANT: diet with the addition of antibiotic; 15 IAP: control diet + 15 mg intestinal alkaline phosphatase kg^{-1} of diet; 30 IAP: control diet + 30 mg intestinal alkaline phosphatase kg^{-1} of diet.
Observed proportions of diarrhoea incidence, followed by different lowercase letters in row, differ from each other by testing the difference between the lsmeans at the 5% probability level.

of IAP in the post-challenge phase of piglets when compared with the other treatments. For the total period, there was treatment effect (P = 0.007), in which piglets that consumed 30 mg of IAP showed lower diarrhoea incidence when compared with those that received 15 mg IAP (Table 6).

4. Discussion

4.1. Counts of intestinal and mesenteric lymph node-adhered microbial populations and pH of digestive tract contents

Enterobacteriaceae population in caecum and MLN was inhibited in piglets fed diets absent of feed additive as observed in piglets fed 30 mg IAP, which may be related to the fact that piglets developed a tolerance to bacterial pathogens or reduced bacterial translocation (Chen et al., 2011). Intestinal alkaline phosphatase has the ability to recompose the gut commensal microbiota in dysbiosis situations, which are often factors related to early-weaned piglets, therapeutic treatment with ANT, and the causes of bowel disease (Alam et al., 2014). Piglets that consumed diets containing ANT suffered a breakdown of the epithelial barrier marked by increased intestinal permeability and Enterobacteriaceae translocation to the MLN (Earley et al., 2015).

Based on the body's defence function and the production of antibodies by the MLN, the presence of microbial populations adhered to the MLN was evaluated with the idea that microorganisms are translocated from the intestine to lymphatic tissues through immune cells, challenging and training the immune system of the animals (Zwirzitz et al., 2019).

Regarding the average LAB count in caecum, this can be attributed to the reduction in IAP activity, being verified only the expression of non-specific alkaline phosphatase isoforms in this portion of the large intestine (Lallès, 2014). The biological action of IAP is non-existent in this portion of GIT when related to the pH of the digestive tract content obtained, which may have contributed to the reduction of LAB in piglets fed 15 or 30 mg IAP. This finding may also be related to the short experimental challenge period used in the present study, which was little able to cause an apparent microbial community disorder (Gao et al., 2013).

The improved microbiota ecosystem, represented by increased LAB and reduction of Enterobacteriaceae in MLN, may be another reason for better intestinal health status (Pan et al., 2017) verified in piglets fed 30 mg IAP. Antibiotic alters the intestinal microbiota and, consecutively, may also affect the corresponding translocation processes, resulting in a state of imbalance between the intestinal microbiota and the host (Zwirzitz et al., 2019). In addition, ANT in diets reduce the abundance of some Gram-positive genera, but do not induce changes in the phylum level in pigs (Kim et al., 2012).

In general, IAP added in the amount of 30 mg modulates the microbiota ecosystem, suppressing the population of Enterobacteriaceae when compared with ANT. When an inflammatory process is induced and a dysbiosis with ATP in the intestinal lumen acts as an inhibitor of the growth of commensal bacteria and the reestablishment of microbiota, the IAP contributes to dephosphorylate this compound, reducing the potential inhibitor of bacterial growth (Malo et al., 2014).

The pH values of large intestine content were lower than other study reports; however, there are no reports of pH values in piglets challenged with F4 receiving IAP in diets. Heo et al. (2013) conducted a review study and reported a pH range in the large intestine similar to our findings. Changes in pH levels are influenced by several factors such as nutritional composition, health status, environmental/housing condition (Jayaraman and Nyachoti, 2017), and measurement site (Heo et al., 2013).

The results of the present study suggest a lower role of pH of the lumen in the modulation of faecal microbiota (Zhang et al., 2010). Greater pH (7.2-7.8) is speculated to provide an ideal environment for ETEC colonisation on the surface of the villi, resulting in early diarrhoea in piglets (Nagy and Fekete, 1999) or when associated with the IAP's ability to modulate the intestinal pH (Brun et al., 2014; Malo et al., 2014). However, the growth of ETEC can be verified in a wide range of pH (4.5 to 9.0) (Gonzales et al., 2013)

and pH 3.0 (Jordan et al., 1999). The reduced pH (<4.5) of the digestive tract content is the appropriate medium for the development of beneficial bacteria, with inhibition of the development of pathogenic bacteria (Suiryanrayna and Ramana, 2015); however, due to the results obtained, it was not possible to corroborate this hypothesis.

4.2. Histopathological description of piglet jejunum

The effect on histopathological characteristics cannot be analysed separately from other concomitant effects, such as that observed for intestinal microbiota. In fact, the observed improvement in health status is probably associated with a multitude of effects at the intestinal level. Histopathological description may be related to the challenge time (Gao et al., 2013) and duration of the inflammatory process.

Apparently, piglets that received IAP in diets showed a tendency to reduce tissue necrosis, and this can be explained by its action to support the immune system (Lallès, 2014), through the dephosphorylation of specific bacterial components, i.e. LPS, CpG DNA, Pam-3-Cys, and flagellin (Chen et al., 2010; Moss et al., 2013) and other molecules, i.e. ATP, ADP, AMP, and UDP (Alam et al., 2014; Malo et al., 2014). In addition, the data suggested that IAP attenuated cell loss due to epithelial desquamation related to the negative control, as well as piglets that received ANT, which may be an indication of reduction of inflammatory alteration. For piglets fed ANT or 30 mg IAP, the findings are consistent when related to hyperaemia with epithelial desquamation and tissue necrosis because the most common occurrence of hyperaemia is observed in acute/pathological inflammation.

When the histopathological description for inflammatory infiltrate was analysed, an occurrence of cell cluster around the epithelium was found to replace functional tissue in piglets of the negative control. We also verified mucus discrimination in piglets that consumed the negative control diet or 15 mg IAP and absence in piglets that received ANT or 30 mg IAP, which can be explained by bacterial challenge (intestinal infection), usually accompanied by diarrhoea and/or damage to the jejunal mucosa (López-Colom et al., 2019).

4.3. Proinflammatory markers on liver and jejunum epithelium and hepatic glycogen reserve

To our knowledge, there seem to be no reports in the literature about studies that determined the COX-2 concentration in piglets fed IAP. Increased COX-2 enzyme activity is related to the stimulation of host cells with bacteria (e.g. bacterial challenge) or bacterial components such as LPS (Lauridsen et al., 2010), inflammatory processes (Kim et al., 2016), and tumour necrosis factor α (Kunanusornchai et al., 2016; Walter et al., 2019). However, the mechanisms of IAP action in piglet diets on COX-2 activity are still limited due to the results of histopathological description in our study.

Kim et al. (2016) tested the effect of acetylsalicylic acid supplementation in piglet diets and also found no differences between treatments for COX-2 content in the liver. However, at least some variation in the COX-2 expression in intestinal samples from 28-d-old piglets can be attributed to the use of antibiotics (Lauridsen et al., 2010), which is not in accordance with the results of this study because all treatments maintained the same variation.

Tumour necrosis factor α was measured as an indicator of proinflammatory systemic response (Ren et al., 2019). We do not know the lack of significant response on TNF-α in piglets fed IAP, although a slight reduction was observed in piglets that received 30 mg IAP. Our findings are in agreement with Beumer et al. (2003), who reported a difference in TNF-α release in piglets treated with LPS compared with piglets treated with LPS + IAP. In our study, the challenge with ETEC did not cause a response of the proinflammatory cytokine TNF-α in the jejunum of piglets. However, TNF-α is involved in cell removal processes of intestinal epithelium (Bischoff et al., 2014) and highly expressed in the chronically inflamed gut (Bischoff et al., 2014). Intestinal damage and exacerbated increase in TNF-α concentration are more consistent in extreme challenges (López-Colom et al., 2019), and the degree and duration of the aggravating effects to tissue are dependent on the post-infection time (Lee et al., 2012). In the

present study, we were unable to verify the role of IAP in reducing the induction of inflammatory responses (Beumer et al. 2003) through attenuating the production of plasma acute phase proteins by the liver (Baumann and Gauldie, 1994) and cell apoptosis (Wan et al., 2019).

The liver plays an important role in regulating glucose production and glycogen synthesis (Fainberg et al., 2012), and hepatic glycogen concentration is related to nutritional quality, piglet body weight, and liver size (Theil et al., 2011), and increased glycogen deposition may be a way to improve short-term survival (Theil et al., 2014). Thus, a reduction in HGR can be attributed as an attempt to keep their vital functions active, and thus increase glycogen depletion (Theil et al., 2011) due to greater glucose turnover rate (Hole et al., 2019).

Toll-like receptors (TLR) play the role of recognizing pathogens and microbial components and trigger an immune response (Xu et al., 2014), but their regulation in studies involving the addition of IAP in diets for weaned piglets challenged with ETEC F4 (K88) are still unexplored. Intestinal alkaline phosphatase can attenuate the increase in TLR4 concentration, since inflammatory processes of pathogenic origin involve increased TLR signalling (Dubreuil, 2017).

In the present study, the *in vivo* challenge model based on ETEC F4 did not cause an immune stimulation through the TLR4 signalling pathway, but some mechanisms in the expression and function of TLR in weaned piglets challenged with ETEC F4 still need further investigations and elucidation (Xu et al., 2014; Luise et al., 2019).

Immune changes in piglets during TLR4 signalling under stress by ETEC F4 in liver damage are poorly known. Proliferative and apoptotic changes during the TLR4 signalling mechanism need further description at the hepatic level (Huang et al., 2017). Even though increased TLR4 signalling may play a key role in liver injury (Huang et al., 2017), piglets maintained a similar response between treatments and had a normal general health status, which can be verified in the production of TNF-α as a pro-inflammatory mediator related to liver injury (Wu et al., 2015).

To our knowledge, there are no reports that analysed the PCNA in studies involving piglets fed IAP and challenged with ETEC F4. In several studies, PCNA is used as a marker of cell proliferation, which encompasses specific proteins or other factors whose presence in active growth and division cells serves as an indicator for such cells (Bologna-Molina et al., 2013). However, the increase in PCNA levels can be induced by growth factors or as a response to damaged DNA, since the intestinal mucosa has a high proliferative rate (Rankin et al., 2004).

The post-weaning period is accompanied by specific changes in the intestinal architecture, which can substantially contribute to cell proliferation, as well as a situation of bacterial challenge, which can be supported by the research conducted by Xia et al. (2018), who reported an increased proliferation of jejunal epithelial cells in infected pigs when compared with the negative control.

However, in this study, the challenge to which the piglets were subjected was poorly able to promote cell regeneration, which implies further investigations to elucidate the mechanisms of action of ETEC F4 (K88) on activation of the PCNA markers. In summary, when the IAP dephosphorylates a bacterial antigen reducing the toxicity of these compounds, there is a reduction in the bacterial potential to induce an inflammatory response (Moss et al., 2013).

4.4. Diarrhoea incidence

The results of the present study for piglets that consumed 30 mg IAP corroborate those presented by Alam et al. (2014), who determined the efficacy of oral supplementation of IAP via drinking water in mice, with positive results for protection against diarrhoea incidence and enteric infections. Intestinal alkaline phosphatase activity in the prevention of diarrhoea incidence has been confirmed, since extensive epidemiological studies demonstrated that the antibiotic-associated diarrhoea, an unwanted consequence of an antimicrobial therapy, is due to changes in the composition and function of the commensal intestinal microbiota, with the consequent overgrowth of opportunistic pathogenic bacteria (Malo et al., 2014).

The effects of IAP obtained in this study are based on its ability to rapidly restore commensal intestinal microbiota in the context of treatment with ANT, which is demonstrated by other studies conducted with mice (Moss et al., 2013; Alam et al., 2014; Malo et al., 2014) and piglets (Beumer et al., 2003). In the present study, the piglets challenged with ETEC exhibited a considerably greater diarrhoea incidence associated with impaired intestinal barrier function (Wan et al., 2019). The F4 inoculation significantly increased the diarrhoea incidence in piglets that did not consume 30 mg IAP. These findings are attributed to the fact that IAP expressed the role of inhibiting adhesion and bacterial internalisation, preventing disruption of barrier integrity and modulating cytokine expression (Ren et al., 2019).

Taken together, the results of the present study indicated a reduced effect of *E. coli* challenge on piglets, justified by the short post-challenge evaluation period as well as the age of inoculation of the bacteria. In addition, experimental conditions such as complex and highly digestible diets may mitigate the potential of additives, and animal daily care may also be an explanation for the lack of more evident results regarding the variables studied.

5. Conclusions

Based on the results, the addition of intestinal alkaline phosphatase in diets does not affect the pH of the digestive tract contents and proinflammatory markers of piglets, but the addition of 30 mg intestinal alkaline phosphatase in diets promotes a suppression of the Enterobacteriaceae population and suggests a possible ability to mitigate intestinal injuries and maintain the homeostasis of the intestinal physiology of piglets through the reduction in diarrhoea incidence and histopathological description, as observed in piglets in the negative control for some of the parameters investigated. In addition, intestinal alkaline phosphatase can be a promising alternative for maintaining intestinal health post-weaning and can possibly partially replace antimicrobials used in weaned piglet diet. The results of our research also suggest future studies with the use of intestinal alkaline phosphatase in diets and different ages of infection in piglets.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

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