UNIVERSIDADE ESTADUAL DO OESTE DO PARANÁ
***CAMPUS* DE MARECHAL CÂNDIDO RONDON**
POSTGRADUATE PROGRAM IN ANIMAL SCIENCE

JANSLLER LUIZ GENOVA

ADDITION OF INTESTINAL ALKALINE PHOSPHATASE IN DIETS AND ITS EFFECTS ON GROWTH PERFORMANCE AND INTESTINAL HEALTH OF WEANED PIGLETS CHALLENGED WITH *Escherichia coli* K88^{+}

Marechal Cândido Rondon
2020

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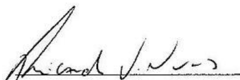

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Marechal Cândido Rondon, 16 de junho de 2020.

DEDICATION

To my Maker and the spirits of light,
To my family, my parents
Luiz Antonio Genova and Vanusa Siqueira dos Santos,
My sister Isabelle Genova,
My grandmother Dinoraide Siqueira,
sublime love, who supported me and gave me strength to overcome all difficulties on this arduous journey ...

To my teachers,
that will always be the foundation of my knowledge, worthy of respect and great value in education ...

To all the friends I've made,
in these academic years ...

With all appreciation and gratitude, I dedicate this work
and all the conquests to come ...

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To the pigs!

BIOGRAPHY

JANSLLER LUIZ GENOVA, son of Luiz Antônio Genova and Vanusa Siqueira dos Santos, was born in Aquidauana - MS, on April 7, 1991.

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EPIGRAPH

"The only place success comes before work is in the dictionary." ***(Albert Einstein)***

"As grandes ideias surgem da observação dos pequenos detalhes." ***(Augusto Cury)***

"The greatest enemy of knowledge is not ignorance; it is the illusion of knowledge." ***(Stephen Hawking)***

"What we know is a drop; what we don't know is an ocean." ***(Isaac Newton)***

ADDITION OF INTESTINAL ALKALINE PHOSPHATASE IN DIETS AND ITS EFFECTS ON GROWTH PERFORMANCE AND INTESTINAL HEALTH OF WEANED PIGLETS CHALLENGED WITH *Escherichia coli* K88^{+}

ABSTRACT – In this study, our aim was to assess the additional effect of intestinal alkaline phosphatase (IAP) in diets on growth performance, diarrhea occurrence (DO), blood metabolites, intestinal histology, relative organ weights, bacterial population counts, pH of digestive tract content, hepatic glycogen reserve (HGR), histopathological description and proinflammatory markers of piglets challenged with enterotoxigenic *Escherichia coli* (ETEC) K88^{+}. A total of 64 crossbred piglets, entire male, weaned at 25-days-old with an average initial body weight of 7.168 ± 0.287 kg were allocated to a randomized complete block design consisting of four treatments repeated twice in the two blocks: control diet (negative control), control diet + antimicrobial growth promoter (AGP, 150 g of tiamulin/ton of diet), control diet + 15 mg IAP/kg of diet and control diet + 30 mg IAP/kg of diet, four replications per block with two piglets per experimental unit. All piglets were orally challenged with 6 mL of a solution containing ETEC K88^{+} (10^6 CFU/mL). Prior to the beginning of the experimental period, was determined the best microencapsulation process of IAP in a model involving adhesion and phagocytic activity of equine bronchoalveolar macrophage. In Exp. I, the variables analyzed were growth performance, DO, blood metabolites (urea, glucose and alkaline phosphatase), intestinal morphometry, relative organ weight and *in vitro* simulation of microencapsulated IAP on pH modulation capacity and its dilution in acidic and basic solution. At 19 experimentation days, six animals per treatment were slaughtered for data collection and biological samples. Exp. II involved the evaluation of the effect of IAP on intestinal health by bacterial populations counts in the intestinal content and adhered to mesenteric lymph node, digestive organ content pH, HGR, proinflammatory markers in the liver and intestinal epithelium and histopathological description of the intestinal epithelium. In pre-starter I phase, piglets that received 30 mg IAP added in the diet or control diet showed better feed conversion rate (P = 0.075) compared to those fed 15 mg IAP. Piglets that consumed 30 mg IAP or control diet showed greater (P = 0.004) average daily body weight gain (ADBWG) in the pre-starter II phase. Piglets fed 15 mg IAP had lower average daily feed intake (ADFI) (P = 0.033) compared to piglets with diets containing AGP. At the total period, there was a difference between treatment, in which the piglets fed 15 mg IAP showed a reduction in ADBWG (P = 0.040) and ADFI (P = 0.092). For the pre-starter II phase, there was a difference (P = 0.044) of treatment, in which the piglets that consumed the diet containing 30 mg IAP showed a 24% improvement

in DO compared to the treatment with 15 mg IAP. We observed the main effect (P = 0.009), with the addition of 30 mg IAP in the post-challenge phase in decreasing piglet DO (5.56%) when compared to those receiving AGP (16.67%). For the total period, piglets that consumed 15 mg IAP showed greater (P = 0.007) DO when compared to those receiving 30 mg IAP. No differences between treatments were obtained in any of the pre- and post-challenge plasma concentration indicators. The spleen relative weight of piglet increased (P = 0.043) in response to 30 mg IAP treatment. The Enterobacteriaceae counts in the cecum content were lower (P = 0.002) in piglets that receiving 30 mg IAP compared with those for AGP treatment. Piglets fed 30 mg IAP presented lower (P = 0.007) Enterobacteriaceae count in the colon when compared to the other treatments. For the Enterobacteriaceae count adhered to the mesenteric lymph nodes (MLN), there was an increase (P = 0.006) in piglets fed diets with AGP. Piglets fed the control diet or AGP showed greater (P = 0.000) lactic acid bacteria (LAB) count in the cecum content. There was a treatment effect (P = 0.013) on LAB count in MLN, in which piglets fed with AGP or that received 30 mg IAP had a greater count when compared to those with 15 mg IAP. The experimental treatments did not influence (P > 0.05) the pH of the digestive tract contents, intestinal morphology, TNF-α, COX-2 activity, TLR4 and proliferating cell nuclear antigen in the jejunum and liver, nor on HGR. Piglets that received 30 mg IAP showed a slight reduction on TNF-α in jejunum (4.17 times) and liver (1.9 times) when compared to piglets in the control group or with AGP, respectively. Based on the present results, the addition of 30 mg IAP in diets improves the growth performance and attenuates the DO in piglets in the post-weaning period. In addition, the results suggest that the addition of 30 mg IAP provides an ability to mitigate intestinal injuries and maintain the homeostasis of the intestinal physiology of piglets.

Keywords: alkaline phosphatase, antibiotics, bacterial challenge, feed additives, immune response, post-weaning diarrhea.

ADIÇÃO DA FOSFATASE ALCALINA INTESTINAL EM DIETAS E SEUS EFEITOS SOBRE O DESEMPENHO ZOOTÉCNICO E SAÚDE INTESTINAL DE LEITÕES DESMAMADOS DESAFIADOS COM *Escherichia coli* K88$^+$

RESUMO – Neste estudo, nosso objetivo foi o de avaliar o efeito adicional da fosfatase alcalina intestinal (FAI) em dietas sobre o desempenho zootécnico, a ocorrência de diarreia (OD), os metabólitos sanguíneos, a histologia intestinal, o peso relativo de órgãos, as contagens de populações bacterianas, pH do conteúdo do trato digestório, a reserva de glicogênio hepático, a descrição histopatológica e marcadores pró-inflamatórios de leitões desafiados com *Escherichia coli* enterotoxigênica (ETEC) K88$^+$. Um total de 64 leitões mestiços, machos inteiros, desmamados com 25 dias de idade e peso corporal inicial médio de 7,168 ± 0,287 kg foram alocados em um delineamento experimental de blocos casualizados completos, consistindo de quatro tratamentos repetidos duas vezes nos dois blocos: dieta controle (controle negativo), dieta controle + promotor de crescimento antimicrobiano (PCA, 150 g de tiamulina/tonelada de dieta), dieta controle + 15 mg de FAI/kg de dieta e dieta controle + 30 mg de FAI/kg de dieta, quatro repetições por bloco com dois leitões por parcela experimental. Todos os leitões foram desafiados via oral com 6 mL de uma solução contendo ETEC K88$^+$ (10^6 UFC/mL). Previamente ao início do período experimental, foi determinado o melhor processo de microencapsulação da FAI em um modelo envolvendo a adesão e a atividade fagocítica do macrófago broncoalveolar de equinos. No Exp. I, as variáveis analisadas foram o desempenho zootécnico, a OD, os metabólitos sanguíneos (ureia, glicose e fosfatase alcalina), a morfometria intestinal, o peso de órgãos relativo e a simulação *in vitro* da FAI microencapsulada sobre a capacidade de modulação do pH e sua diluição em solução ácida e básica. Aos 19 dias de experimentação, seis animais por tratamento foram abatidos para coleta de dados e amostras biológicas. O Exp. II envolveu a avaliação do efeito da FAI sobre a saúde intestinal pela contagem de populações bacterianas no conteúdo dos intestinos e aderidas aos linfonodos mesentéricos, pH do conteúdo do trato digestório, RGH, marcadores pró-inflamatórios no fígado e no epitélio intestinal e descrição histopatológica do epitélio intestinal e fígado. Na fase pré-inicial I, os leitões que receberam 30 mg de FAI adicionada na dieta ou do grupo controle mostraram melhor taxa de conversão alimentar (P = 0,075) em comparação aos alimentados com 15 mg de FAI. Houve efeito (P = 0,004) de tratamento sobre o ganho de peso corporal diário médio (GPCDM) na fase pré-inicial II. Os leitões que foram alimentados com 15 mg de FAI tiveram menor consumo de ração diário médio (CRDM) (P = 0,033) em comparação aos leitões com dietas contendo PCA. No período total, houve diferença sobre o

GPCDM (P = 0,040) e CRDM (P = 0,092). Para a fase pré-inicial II, houve diferença (P = 0,044) de tratamento, em que os leitões que consumiram a dieta contendo 30 mg de FAI apresentaram melhora de 24% na OD em comparação aos do tratamento com 15 mg de FAI. Nós observamos o principal efeito (P = 0,009), com a adição de 30 mg de FAI na fase pós-desafio na redução da OD de leitões (5,56%) quando comparado aos que receberam PCA (16,67%). Para o período total, houve efeito (P = 0,007) dos tratamentos, em que os leitões que consumiram 15 mg de FAI mostraram maior OD quando comparado aos recebendo 30 mg de FAI. Não foram obtidas diferenças entre os tratamentos em qualquer um dos indicadores de concentração plasmática pré e pós-desafio. O peso relativo do baço de leitões aumentou (P = 0,043) em resposta ao tratamento com 30 mg de FAI. As contagens de Enterobacteriaceae no conteúdo do ceco foram menores (P = 0,002) para os leitões que receberam 30 mg de FAI em comparação com aqueles do tratamento com PCA. Os leitões alimentados com 30 mg de FAI apresentaram menor (P = 0,007) contagem de Enterobacteriaceae no cólon quando comparados aos demais tratamentos. Para a contagem de Enterobacteriaceae aderidas aos linfonodos mesentéricos (LM), houve redução (P = 0,006) nos leitões que ingeriram dietas com PCA. Os leitões alimentados com a dieta controle ou com PCA mostraram maior (P = 0,000) contagem para bactérias ácido lácticas (BAL) no conteúdo do ceco. Houve efeito (P = 0,013) de tratamento sobre a contagem de BAL nos LM, em que os leitões alimentados com PCA ou que receberam 30 mg de FAI apresentaram maior contagem quando comparados aos com 15 mg de FAI. Os tratamentos experimentais não influenciaram (P > 0,05) o pH do conteúdo de órgãos digestórios, a morfologia intestinal, a concentração de TNF-α, de TLR4 e do antígeno nuclear de proliferação celular, e a atividade de COX-2 no jejuno e fígado, nem sobre a RGH. Os leitões que receberam 30 mg de FAI apresentaram ligeira redução do TNF-α no jejuno (4,17 vezes) e no fígado (1,9 vezes) quando comparados aos leitões do grupo controle ou com AGP, respectivamente. Com base nos resultados, a adição de 30 mg de FAI nas dietas melhora o desempenho zootécnico e atenua a OD em leitões no período pós-desmame. Além disso, os resultados sugerem que a adição de 30 mg de FAI proporciona uma capacidade de mitigar lesões intestinais e manter a homeostase da fisiologia intestinal de leitões.

Palavras-chave: fosfatase alcalina, antibióticos, desafio bacteriano, aditivos alimentares, resposta imunológica, diarreia pós-desmame.

LIST OF ABBREVIATIONS

ADBWG - Average daily body weight gain

ADFI - Average daily feed intake

ADP - Adenosine diphosphate

AGP - Antimicrobial growth promoter

AIC - Akaike information criteria

AM - Alveolar macrophage

AMP - Adenosine monophosphate

ANCOVA – Analysis of covariance

ANOVA - Analysis of variance

AP - Alkaline phosphatase

ATP - Adenosine triphosphate

BAL - Bronchoalveolar lavage

BHI - Brain heart infusion

cAMP - Cyclic adenosine monophosphate

CD - Crypt depth

CD-14 - Cluster of differentiation 14

CFU - Colony forming unit

cGMP - Cyclic guanosine monophosphate

COX-2 - Cyclooxygenase-2

DNA - Deoxyribonucleic acid

DO - Diarrhea occurrence

EHEC - Enterohemorrhagic *E. coli*

EIEC - Enteroinvasive *E. coli*

EMB – Eosin methylene blue

ENS - Enteric nervous system

EOs - Essential oils

EPEC - Enteropathogenic *E. coli*

ETEC - Enterotoxigenic *E. coli*

ExPEC - Extraintestinal pathogenic *E. coli*

FBW - Final body weight

FCR - Feed conversion rate

GBA - Gut-brain axis

GIT - Gastrointestinal Tract

GLM - Generalized linear model

IAP - Intestinal alkaline phosphatase

IBW - Initial body weight

IL - Interleukins

LAB - Lactic acid bacteria

LI - Large intestine

LPS - Lipopolysaccharide

LT - Labile toxin

MLN - Mesenteric lymph nodes

mRNA - Messenger ribonucleic acid

MRS - Man, Rogosa and Sharpe

PA - Palmitic acid

PA - Phytogenic additives

PBS - Phosphate buffered saline

PGE - Propylene glycol ester

pH – Potential of hydrogen

PRRs - Pattern recognition receptors

PWD - Post-weaning diarrhea

PYY - Peptide YY

RS - Rice starch

SEM - Standard error of the mean

SI - Small intestine

ST - Thermostable peptide toxin

STEC - Shiga toxin-producing *E. coli*

TLRs - Toll-like

TNF - Tumor necrosis factor

TRPV5/6 - Transient receptor potential ion channel subfamily V and VI

UDP - Uridine diphosphate

VH - Villus height

VTEC - Verotoxin

ZO - Zonula occludens

SUMMARY

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1 INTRODUCTION

Recently, the “gastrointestinal tract health” has been greatly investigated despite the lack of a clear definition of the term or its etiology, although in general terms, the “intestinal health” encompasses a series of physiological and functional characteristics (PLUSKE et al., 2018). Any discussion of intestinal health in the post-weaning period should include the potential impacts of enterotoxigenic *E. coli* (ETEC) strains, which is a major challenge for piglets (YI et al., 2016; GRESSE et al., 2017; LUISE et al., 2019). These bacteria adhere to the small intestine epithelium and although ETEC does not directly induce harmful morphological changes, they secrete enterotoxins impairing enterocyte functions by increasing fluidity and reducing water absorption (SUN & KIM, 2017).

Thus, in recent decades, a great deal of research has been devoted to the development of alternatives to antibiotics to maintain the health and growth performance of pigs. The most widely researched alternatives include organic and inorganic acids, high levels of zinc oxide, essential oils, herbs and spices, some types of prebiotics, probiotics, bacteriophages, antimicrobial peptides, feeding strategies and available handling that influence or intend to influence different aspects of intestinal health (ADEWOLE et al., 2016; JAYARAMAN & NYACHOTI, 2017; LIU et al., 2018).

Current studies have been reported the potential therapeutic effect of the alkaline phosphatase enzyme in decreasing inflammatory bowel disease (BILSKI et al., 2017) and metabolic diseases such as insulin resistance (GUL et al., 2017), in reducing vascular calcification and improving cardiovascular outcomes in patients with chronic kidney disease or type 2 diabetes mellitus (HAARHAUS et al., 2017), use in the diagnosis of bone tumors such as osteosarcoma (AGUSTINA et al., 2018; GU & SUN, 2018), as a prognostic marker for patients with prostate cancer (HEINRICH et al., 2018) and upper tract urothelial carcinoma (TAN et al., 2018).

Besides that, previous studies have demonstrated the effect of intestinal alkaline phosphatase (IAP) isoform on improving the intestinal health of animals (LALLÈS, 2014; MELO et al., 2016; BILSKI et al., 2017; RADER, 2017), including: dephosphorylation of bacterial lipopolysaccharide (LPS), reducing the toxicity of LPS and, consequently, reducing inflammatory processes in the gut (BEUMER et al, 2003; BATES et al., 2007); dephosphorylation of luminal ATP, acting as a prebiotic in regulating the growth of commensal bacteria (ALAM et al., 2013; MALO et al., 2014); modulation of intestinal pH (AKIBA et al., 2007); inorganic phosphate homeostasis (SASAKI et al., 2018);

In this same line of reasoning, it is possible that IAP is an alternative to antibiotics with potential effect in the post-weaning period of piglets. Our hypothesis was that the addition of IAP to piglet diets would improve intestinal health by reducing inflammatory processes in the intestine and consequently a benefit on growth performance in the nursery phases, being an effective alternative to the traditional antibiotics used as performance enhancers. Thus, the aim of this study was to evaluate the effect of dietary intestinal alkaline phosphatase on growth performance, blood metabolites and intestinal health of weaned piglets challenged with *Escherichia coli* K88^{+}.

2 LITERATURE REVIEW

2.1 The use of feed additives in the piglet nutrition with the role of a healthy gastrointestinal tract

Recent studies use a range of products and handling alternatives such as feed additives (Table 1) and feeding strategies (ADEWOLE et al., 2016; LIAO & NYACHOTI, 2017; ZHAI et al., 2018), in order to improve the growth performance, minimizing the use of antimicrobial growth promoters (AGP) and inaccessible feed ingredients.

Table 1. Current results with the addition of feed additives in piglet diets.

Items	Body weight (kg)	Days of age	Additive dose	Main effects	References
Enzyme (*Buttiauxella* phytase)	11.0 ± 1.5	21 and 28	500, 1000 or 2000 FTU/kg	increased ADG and G:F, linear response on nutrient digestibility and reduced P and Ca^{2+} excretion	Dersjant-Li et al. (2017)
Enzyme (carbohydrase blend: cellulase, β-glucanase, and xylanase)	6.43 ± 0.06	-	0.01%	enhanced growth rate, improved small intestinal barrier integrity and reduced immune activation	Li et al. (2018)
Enzyme (xylanase)	6.43 ± 0.06	-	0.01%	reduced ATTD of NDF and ADF	Li et al. (2018)
Enzyme (phytase)	5.6 ± 0.5	-	500 or 2000 FTU/kg	2000 FTU/kg: increased ADG, ADFI, feed efficiency,	Lu et al. (2019)

				plasma inositol concentration and expression of GLUT4	
Essential oil (thymol 50% and carvacrol 50%)	6.5 ± 0.9	-	100 mg/kg diet	decreased the intestinal oxidative stress and influenced microbial populations	Wei et al. (2017)
Essential oil (thymol 25% and carvacrol 25%)	8.64 ± 0.33	-	30 mg/kg	improved nutrients digestibility, intestinal morphology, digestive enzymes and ↑ Lactobacilli counts of feces	Xu et al. (2018)
Organic acid (sodium butyrate salt)	8.3 ± 0.32	24	2.1 g/kg diet	reduced colonization and shedding of *Salmonella*	Barba-Vidal et al. (2017a)
Organic acid (SCFA: formic, acetic and propionic acid combined with MCFA)	8.63 ± 1.56	-	2000 or 3000 mg/kg	reduced the incidence of diarrhea and fecal *E. coli* counts, ↑ nutrients digestibility and positive effects on serum immunity	Long et al. (2018)
Organic acid (MCFA and AO)	8.09 ± 0.11	28	0.2%, 0.4% or 0.6%	combinations of organic acids could improve growth performance, reduce post-weaning diarrhea, and enhance serum immunity	Han et al. (2018)
Organic acid (fumaric	6.54 ± 0.78	-	0.1% or 0.2%	improved growth	Upadhaya et al.

acid 17%, citric acid 13%, malic acid 10% and 1.2% MCFA)				performance and nutrient digestibility	(2018)
Organic acid (benzoic acid 50%, calcium formate 3%, fumaric acid 1%)	8.64 ± 0.33	-	1.5 g/kg	improved nutrients digestibility, fecal score, intestinal morphology, ↑ Lactobacilli counts of feces and butyric and valeric acid concentration	Xu et al. (2018)
Plant extract (*Eucommia ulmoides*)	7.22 ± 0.34	21 ± 2	0.5% or 6%	improved growth performance, jejunal morphology and changed colonic microbial composition	Peng et al. (2019)
Prebiotic (galacto-oligosaccharides, GOS)	1.55 ± 0.05	-	10 mL (1 g GOS/kg BW)	increased ADG, decreased crypt depth, increased the jejunal lactase, maltase and sucrase activity, facilitated the mRNA expression of SGLT1 and GLUT2	Tian et al. (2018)
Prebiotic (β-glucans, glucomannans and MOS)	6.32 ± 0.10	21	1000, 2000, or 3000 mg/kg	3000 mg/kg reduced the jejunal villus density increased spleen weight	Anjos et al. (2019)

Prebiotic (β-glucan)	7.3 ± 0.2	-	0.25 g/kg	did not improve gut health	Mukhopadhya et al. (2019)
Probiotic (*Bacillus licheniformis*)	8.3 ± 0.32	24	10^9 CFU/kg diet	reduced colonization and shedding of *Salmonella,* effect on behavioral displays	Barba-Vidal et al. (2017a)
Probiotic (*Bifidobacterium longum* subsp. *infantis* CECT 7210)*	7.9 ± 0.05 and 6.8 ± 0.19	24 ± 4 and 21 ± 2	10^9 CFU which was supplemented in a 2 mL solution	reduced the fecal excretion of *Salmonella Typhimurium* and the mucosal colonization of coliforms in the ETEC K88 trial, produced a stimulation of the intestinal immune system	Barba-Vidal et al. (2017b)
Probiotic (*Bacillus licheniformis and Saccharomyces cerevisiae*)	4.9 ± 0.4	21 ± 3	500 mg/kg diet	alleviated the ↑ in the endotoxin and diamine oxidase concentration, and cecal *E. coli* count	Pan et al. (2017)
Probiotic (*Bacillus amyloliquefaciens)*	14.57 ± 0.25	-	2 x 10^8 CFU/kg diet	increased the activities of amylase, disaccharides and Na^+/K^+-ATPase, maintained the intestinal integrity and decreased activity of diamine oxidase	Hu et al. (2018)
Probiotic (*Clostridium butyricum*)	-	28	2 x 10^6 or 5 x 10^5 CFU/g diet	did not significantly reduce fecal excretion,	Peeters et al. (2019)

	serological response and intestinal carriage

* = Two experiments, BW = body weight, ADG = average daily gain, G:F = gain:feed, ADFI = average daily feed intake, CFU = colony-forming units, GLUT2 = glucose transporter type 2, GLUT4 = glucose transporter type 4, SGLT1 = sodium glucose co-transporter 1, ATTD = apparent total tract digestibility, NDF = neutral detergent fiber, ADF = acid detergent fiber, FTU = phytase units, AO = acid organic, SCFA = short chain fatty acids, MCFA = medium chain fatty acids, mRNA = messenger RNA.

The wide number of feed additives for use as alternatives or substitutes to AGP that have been evaluated in pig nutrition generally aims to: improve immune responses and effective immune status (e.g. immunoglobulin, ω-3 fatty acids, yeast-derived β-glucans); reducing pathogenic microorganisms load at gastrointestinal tract (e.g. organic and inorganic acids, higher levels of zinc oxides, essential oils, herbs and spices, some types of prebiotics, probiotics, bacteriophages, antimicrobial peptides); stimulate the establishment of mutualistic or commensal microorganisms in the GIT (e.g. probiotics and some types of prebiotics) and/or stimulate digestive process (e.g. butyric acid, gluconic acid, lactic acid, glutamine, threonine, cysteine, nucleotides, essential oils, herbs and spices, and enzymes) (DE LANGE et al., 2010).

Therefore, Adewole et al. (2016) mentioned that feed additives such as zinc oxide, copper sulfate, spray-dried porcine plasma, egg yolk antibodies and mannanoligosaccharides have been used by several researchers and in research models for the purpose to improve growth and also to prevent disease, but the effectiveness of these additives depends mainly on the amount added to the diet.

Along the same line of reasoning, Celi et al. (2017) reiterated that the reason for this inconsistency may be that the effectiveness of each additive depends on diet (e.g. composition, diet processing and feeding methods), colonization and associated succession of microbial populations, stress and genetics. Therefore, it is not possible to recommend a specific additive that has positive effects on all diets, but it is likely that if no AGP is used at least some additives are beneficial in diets fed to piglets (LIU et al., 2018).

Pluske et al. (2018) reported that additives are predominantly characterized not only by their different modes of action, but also by the variation in responses obtained when added to pig diets. This variation is presumably a consequence, in part, of the many different management conditions in which pigs are subjected, which in turn influence factors such as microbiota composition and intestinal mucosal immunity.

Regarding the use of essential oils (EOs) in pig nutrition, Stevanović et al. (2018) argued that the chemical composition of the EOs depends on plant genetics, plant growth conditions, harvest developmental stage and extraction processes of active compounds. Moreover, their biological effects are influenced by the interaction of phytochemicals and their bioavailability in the animals' GIT. In addition, research with EOs should focus on reliable methods to identify and control the quality and effects of EOs.

Additional studies should be conducted on phytogenic additives (PAs) or simply phytobiotics in order to verify effects on the intestinal health of piglets, as a systemic approach

is required to explain the role of these PAs in terms of type and dose of each additive. Besides that, the potential benefits of phytobiotics may differ due to the wide variation in the plants diversity, composition and active principles, which results in difficulty in comparing the efficiency of different PAs (SURYANARAYANA & DURGA, 2018).

Regarding to prebiotics, Liu et al. (2018) further highlighted a possible positive impact on the immune and microbiological system, but additional research is needed to document these effects. It is also possible that the use of prebiotics, direct-feeding microorganisms, yeast and nucleotides may have positive impacts on the growth performance of pigs, but the results have been less consistent, as the efficiency of each additive depends on diet and also on the health status of the animals (LIU et al., 2018). There is a need for further research in this area on the interaction with other additives and mechanisms of action.

The aforementioned authors have also reported that, despite many years of research, the exact mode of action of the dietary acidifiers has not yet been fully elucidated and further investigation are needed. However, according to Kil et al. (2011) and Pearlin et al. (2020), the most reported mechanisms include GIT pH reduction, and thus can affect the growth of pathogenic bacteria, besides having a role in improving the nutrient digestion. Although there are other possible mechanisms of action of acidifiers in the literature, further evidence is needed to support these findings.

Corroborating previous reports, the addition of exogenous enzymes in piglet diets has focused on understanding and determining the effects on growth performance parameters and nutrient digestibility. However, there is a need to clarify the effects of feed enzymes on aspects involving intestinal health (BEDFORD & COWIESON, 2012). In this sense, several mechanisms of how enzymes act on the intestinal microbiota have been clarified (KIARIE et al., 2013), but the extent to which these effects may contribute to the overall health of GIT is unknown.

Thus, there is a need for additional and other research in order to validate the findings. An important point to be discussed in this review is the role of feed enzymes as possible alternatives to AGP (CHENG et al., 2014), as exogenous enzymes can improve intestinal stability by reducing substrates for putrefying organisms, by increasing substrates for beneficial fermentative organisms and the ability of the gut to defend itself against unwanted bacterial ingress (CELI et al., 2017).

In this perspective, the potential use of enzymes in order to promote an improvement in the intestinal health of piglets and to reduce diarrheal events during the post-weaning phase require studies and information. Melo et al. (2016) reported that, although some studies have

verified the potential roles of IAP on intestinal health, investigations on the exogenous effects of IAP or feed additives modulating IAP expression and activity are still needed. Therefore, the alternatives research to AGP and the development of new efficient and safe alternatives will be a long process (CHENG et al., 2014).

Alternatives to AGP reported in this review are in constant research and it is possible that in future others studies and methods will be developed with the purpose of evaluating mechanisms that have not yet been researched, such as the *in vivo* use of bioinspired synthetic peptides in toxins against bacteria and other pathogens and antimicrobial photodynamic inactivation. In summary, inconsistent results obtained with feed additives may be due to differences in the pig age, health status, environmental conditions or available handling.

2.2 Physiological mechanisms and the health of the gastrointestinal tract

The health of the GIT ("intestinal health") is a commonly used term and is a matter of enormous interest currently research with piglets, but it generally does not have a precise and unifying meaning or etiology (PLUSKE et al., 2018). "Intestinal health" is a reflection of several interactions between the animal and its environment (Figure 1).

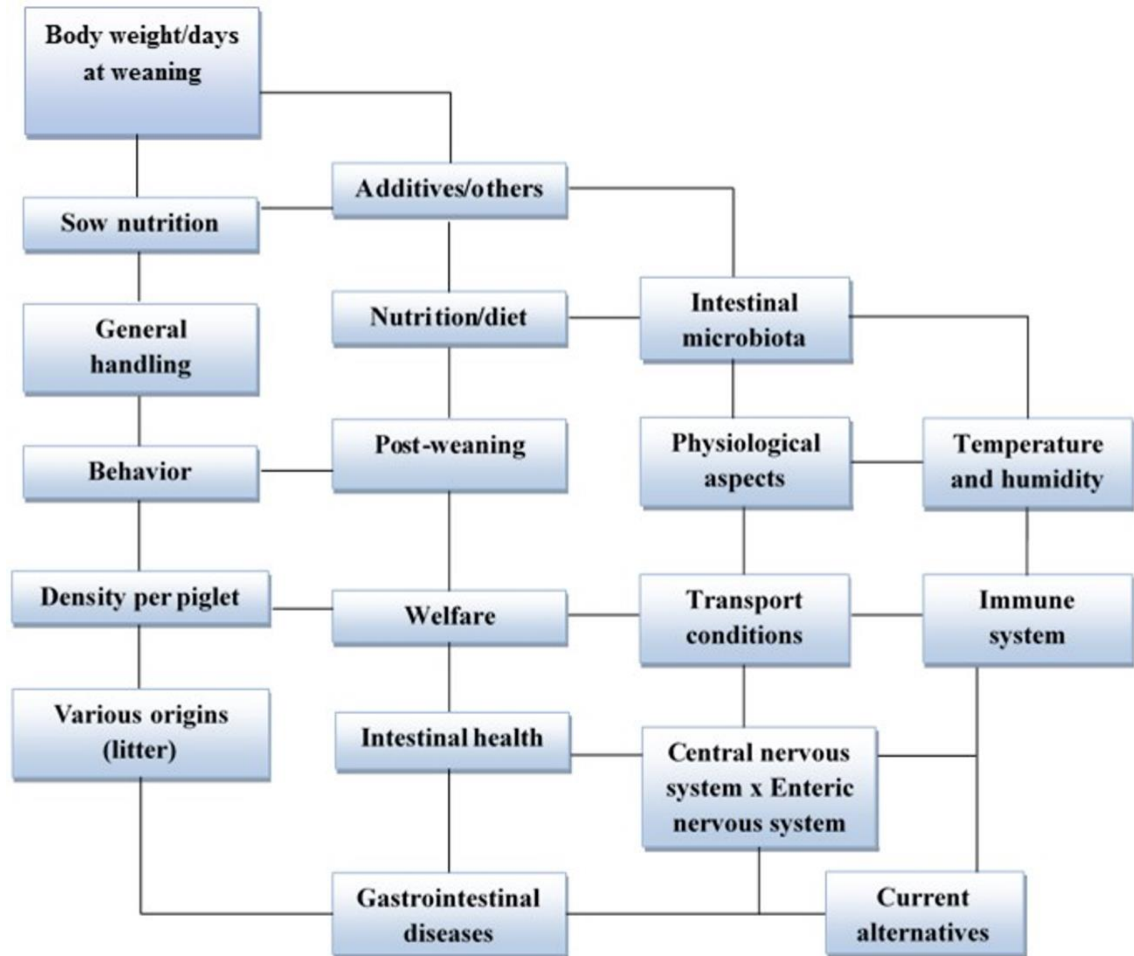

Figure 1. Fundamental components for promoting intestinal health of piglets during the nursery phase.

According to Kogut & Arsenault (2016), GIT health is defined as the absence and/or prevention of diseases for the animal to be able to perform its physiological functions to resist exogenous and endogenous stressors. Corroborating this idea, Bischoff (2011) defined five main criteria that could form the basis of a comprehensive definition of intestinal health, which would be: effective digestion and absorption of dietary nutrients; absence of IBD or gastrointestinal; optimal and stable intestinal microbiota/microbiome; effective and active immune status, and indicative of animal welfare.

In this sense, the abrupt post-weaning period in piglets not only causes structural and functional changes accentuated in the small bowel, but also contributes to an inflammatory bowel process that, in turn, compromises villus and crypt architecture (PLUSKE et al., 2018). Jayaraman & Nyachoti (2017) argued that inadequate management practices may result in reduced feed intake, stress and predisposition to disease conditions, consequently affecting intestinal health and performance of weaned piglets.

However, the functions of the GIT extend beyond the processes associated with feed intake, nutritional particle digestion and concomitantly active or passive absorption and intestinal barrier function, since the GIT plays an important role in the regulation of epithelial, physiological and immunological functions. Celi et al. (2017) suggested that the main components of GIT functionality are effective diet, integrity and function of the gastrointestinal barrier, host interaction with the resident microbiota, effective nutrient digestion and absorption and active immune status.

Other research has reported that the association between the enteric nervous system (ENS) and the upper centers via the parasympathetic nervous system and/or the endocrine system also plays a fundamental role in the animal welfare, intestinal health and the structure/integrity and function of the GIT (MOESER et al., 2017), i.e., the complex interactions that occur in the GIT between nutrition, the mucosa (epithelium) of the GIT and intestinal microbiota are essential to affect intestinal health (PLUSKE et al., 2018), although the precise mechanisms of how the GIT microbiome contributes to changes in behavior and central nervous system effects are less evident (FOSTER et al., 2017).

In general, GIT health is a complex system that includes several factors, but it is of paramount importance to highlight gastrointestinal barriers (e.g. GI epithelium barrier, GI immune system barrier and ENS barrier), GIT microbiota/microbiome and nutritional management (ADEWOLE et al., 2016; CELI et al., 2017; JAYARAMAN & NYACHOTI 2017; MOESER et al., 2017; BARBA-VIDAL et al., 2018; LIU et al., 2018; PLUSKE et al., 2018).

As previously mentioned, GIT is a very complex, dynamic and constantly changing organ, such as in the microbiota composition, rapid and accentuated changes in the digestive barrier, absorptive and immune functions. In addition, GIT is composed of the GI barrier, in which it is formed by a multilayered system of host defense mechanisms, provided by the gut epithelial cells and components of the immune nervous system and ENS (MOESER et al., 2017).

The intestinal barrier is mainly formed by a layer of epithelial cells attached by tight-junction proteins called tight junctions, consisting mainly of transmembrane protein complexes (i.e. claudins and occludins) and cytosolic proteins ZO (i.e. junctional adhesion molecule, ZO-1, ZO-2 and ZO-3) (LIU et al., 2018). ZO-1 and occludin are key tight-junction proteins, and levels of these proteins are consistently associated with intestinal barrier function (SONG et al., 2015).

As described by Pluske et al. (2018), the intestinal barrier function along with the

mucosal immune system are continually challenged by external (i.e. diet provided) and internal (i.e. intestinal microbiota) factors, as well as several cell types such as dendritic cells, lymphocytes (adaptive or acquired immune system), macrophages and cytokines (innate or non-specific immune system) have evolved to perform important roles in regulating the communication between the GIT microbiome and its mucosal immune system.

Moeser et al. (2017) reported that the intestinal epithelial barrier is also supported by other types of specialized epithelial cell, such as goblet cells, which provide a protective mucous layer and Paneth cells that secrete antimicrobial peptides. In addition, enteroendocrine cells play important roles in the pathogen detection and can synthesize and release neuropeptides (i.e. serotonin and Peptide YY, PYY), which have a diverse range of physiological functions from pathogen defense to metabolic regulation of appetite (DUCA et al., 2013; MOESER et al., 2017). In short, intestinal epithelial cells act as immune sentinel cells, recognizing pathogenic signaling molecules and secreting interleukins (IL) and growth factors (i.e. IL-17A, IL-33, IL-23 and transforming growth factor-β), which have important immunomodulatory properties (SCHIERING et al., 2014; MOESER et al., 2017; PLUSKE et al., 2018).

Another mechanism that acts directly to promotes this intestinal health is the ENS barrier, through the constant release of a series of neurochemicals substances, plays a central role in the motility, secretion and absorption of the gut and the modulation of epithelial permeability. Furthermore, the nervous system is also one of the main regulators of local gastrointestinal systemic and immune responses via neuroimmune synapses and can modulate the sensitivity and adherence of bacterial toxins (MOESER et al., 2017). There is a growing interest in studying neuronal-immune communication as a way to explain mechanisms of gastrointestinal diseases.

From this point of view, it is evident that numerous factors influence the diversity and activity of the GIT microbiota, including colonization and associated succession of microbial populations (PLUSKE et al., 2018), as well as the interaction between the microbiota and the gut-brain axis (GBA), through the signaling from gut microbiota to brain, as well as in the opposite direction by means of neural, endocrine, immune and humoral connections (CARABOTTI et al., 2015).

When the intestinal health approach is related to nutritional management, it is important to keep in mind that associated with nutrition can modulate immune function in the GIT through several distinct mechanisms such as cytokine production and regulation of the gut barrier function (CELI et al., 2017). Furthermore, it may influence the composition and metabolic activity of the GIT microbiota as a consequence of changes in substrates available for microbial

fermentation (YEOMAN & WHITE, 2014).

Studies conducted with other species showed that dietary components may alter intestinal permeability (DAVID et al., 2014; KELLY et al., 2015). In particular, high-fat diets are associated with a greater lipopolysaccharide (LPS) translocation through the intestinal wall (MOREIRA et al., 2012). In addition, meals rich in fiber and fruit have been shown to reduce meal-induced increases with high fat/carbohydrate content at LPS plasma levels, inflammatory response and Toll-like receptors (TLR) 2 and 4 expression (GHANIM et al., 2009).

In this context, the effects of diet on gastrointestinal health can be directed to different functions of GIT. At the same time, the effects of the central nervous system on the microbiota composition are probably mediated by a disturbance of the normal luminal/mucosal habitat that can also be restored by diet (CARABOTTI et al., 2015). Ultimately, "intestinal health" represents the outcome of GIT in response to its ability to react and adapt to the insults and challenge models it encounters (ADEWOLE et al., 2016; PLUSKE et al., 2018).

Moreover, the gut microbiota (or microbiome, representing the genomic information of the microbial, intestinal ecology) represents a compromise between useful barrier functionality, synthesis of beneficial nutrients and proteins, better energy acquisition, action on the deleterious effects of inflammation and subclinical/clinical pathologies (CELI et al., 2017), influence on the functional diversity of B and T cells, with emphasis on differentiation of B and T cells IgA-producing that carry the CD4 antigen (HONDA & LITTMAN, 2016).

Finally, studies are needed to clarify the role of the GIT microbiota in the relationships between animal nutrition (diet), physiology (digestion and absorption), health (immunology) and welfare (GBA). Celi et al. (2017) mentioned that the multifaceted and widely unknown interactions between microbial populations, and the GIT microbiota and host, add another complexity level to this research area.

2.3 Potential impacts of *Escherichia coli* enterotoxigenic strains

Post-weaning diarrhea (PWD) is one of the major concerns related to the intestinal health of piglets, which is often caused by enterotoxigenic *E. coli* (ETEC) infections, mainly including F4 (K88)$^+$ and F18$^+$. The main virulence factors of ETEC are adhesins (fimbria or pili) and enterotoxins (peptides or proteins), where the most common types of fimbriae in piglets ETEC are F18$^+$ and F4$^+$ (SUN & KIM, 2017; LUISE et al., 2019).

According to Abubakar et al. (2017) there are five common, antigenically distinct types of pili found in pigs: F4 (K88), F5 (K99), F41, F6 (987P) and F18, in which the first four types

of pili mediate adhesion in neonates. F18 is not associated with neonatal colibacillosis, but is common in post-weaning colibacillosis, as is F4 (Nagy et al., 1997; FAIRBROTHER et al., 2005; FAIRBROTHER & NADEAU 2019).

Smeds et al. (2001, 2003) identified five genes as members of the operon encoding F18 fimbriae: fedA (main pilus subunit), fedB (usher protein), fedC (chaperone), fedE (minor protein of unknown function) and fedF (adhesin). In this sequence, F4 are flexible fimbriae that occur as variants of F4ab, F4ac, or F4ad, in which F4ac is the dominant type, worldwide (FAIRBROTHER et al., 2005). The genes encoding F4ab and F4ac were analyzed and showed to be organized into an operon of 10 genes that differ between variants of the *faeG* gene encoding adhesin. In contrast to F18 fimbriae, in which adhesin is a minor protein, distinct from the major fímbrial subunit, FaeG is the major protein of the fímbrial subunit and adhesin (JOENSUU et al., 2004; FAIRBROTHER et al., 2005).

However, *E. coli* variants may differ in their adhesins as well as in the elaborate enterotoxins. ETEC is able to synthesize a wide range of enterotoxins, including the heat-labile toxin I and II (LTI and LTII), thermostable peptide toxins A and B, STa and STb, respectively (FRANCIS, 2002; ABUBAKAR et al., 2017), while Shiga toxin-producing *E. coli* (STEC) synthesizes verotoxin (VTEC) or also called Shiga toxin, Stx (CHOI et al., 2001b; FAIRBROTHER et al., 2005; ABUBAKAR et al., 2017; SUN & KIM, 2017). The first three types of toxins act locally, causing hypersecretion of fluid from the bowel, while VTEC is responsible for the systemic vascular effects of edema disease (NAGY & FEKETE, 1999; CHOI et al., 2001b).

Another important point is about the classification of *E. Coli* and Fairbrother & Nadeau (2019) state that pathotype is the term used to classify *E. coli* by its virulence factors and that the major classes identified include: Shiga toxin-producing *E. coli* (STEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC) and extraintestinal pathogenic *E. coli* (ExPEC). In addition, Choi et al. (2001a) reported on enteroinvasive *E. coli* (EIEC). Other strains have the ability to erode the epithelium, causing attaching-and-effacing (A/E) lesions, being called attaching and effacing *E. coli* (AEEC) (FRÖHLICHER et al., 2008).

In addition, Fairbrother & Nadeau (2012) observed that the best approach to classifying *E. coli* is by serotyping in association with virulent strains. However, according to these authors, only a small percentage of organisms are typifiable based on O, K, H, and F antigens, and only about 175O, 80K, 56H, and more than 20F antigens have been officially recognized to date, based on proven or suspected pathogenicity of isolated *E. coli*. Among these, the main serogroup of *E. coli* causing PWD in piglets is O149 (SUN & KIM, 2017). Other serogroups

that frequently cause PWD are O8, O138, and O141 (NAGY et al., 1997; NAGY & FEKETE, 2005; FRANCIS, 2002; FRYDENDAHL, 2002).

Regarding to the *E. coli* pathogenesis, the cause of PWD may also be related to the piglet's genetic ability to present or not receptors for the different types of adhesins. F4 receptors are fully expressed from birth to physiological maturity of the piglet (FAIRBROTHER et al., 2005), while the F18 receptor is not fully expressed until about 20-days-old in piglets (NAGY et al., 1992). The colonization degree of ETEC is also associated with the small bowel segment, in which fimbriae F5, F6 and F4 colonize mainly in the distal jejunum and ileum, while F4 has the ability to colonize throughout the jejunum and ileum (SUN & KIM, 2017).

As a consequence, the action mechanism of LT and ST enterotoxins is to impair epithelial cellular function of the small bowel, resulting in increased water and electrolyte secretion, especially sodium and chlorine, with decreased fluid absorption and increased dehydration (FRANCIS, 2002; ZHANG et al., 2010; SUN & KIM, 2017), and in certain cases leading the piglet to sudden death (GUPTA et al., 2008). More specifically, LT induces secretion of ions (chloride, sodium and bicarbonate) and water in the lumen through its B subunits that connect to the mucosal cells GM1 receptor, which causes activation of the adenylate cyclase system by increasing the cAMP concentration (FAIRBROTHER et al., 2005; SUN & KIM, 2017).

In turn, protein kinase A is stimulated by cAMP by phosphorylation the cystic fibrosis transmembrane conductance regulator (CFTR), causing Cl^{-} secretion from the luminal surface of the enterocytes. STa inhibits the absorption of sodium and chloride ions from the intestinal lumen into the epithelial cell through the guanylate cyclase system, inducing intracellular accumulation of cGMP, which results in fluid retention (ABUBAKAR et al., 2017; SUN & KIM, 2017). However, the molecular mechanism of how STb acts is not well understood (SUN & KIM, 2017).

Finally, AGP used to be the most effective way to prevent PWD, however, with increasing bacterial resistance to antibiotics, alternatives to AGP are urgently needed. Thus, feed additives such as IAP can prevent ETEC-associated PWD and improve the intestinal health of post-weaning piglets.

Research that verifies the additional effect of IAP on the piglet diets post-challenge with ETEC is scarce and needs further investigation. Finally, ETEC strains are directly related to the post-weaning period, mainly to PWD events. IAP has a potential effect on the intestinal health of piglets, but it is not yet known to what extent its effect is validated because the intestinal health in this transition period is modulated by several stressors. So far, there are many

alternatives have been investigated for antibiotic withdrawal, but this process may take a long time. There is not a single ingredient or product capable of resolving all intestinal health issues, but rather the association of several factors. The nutrition aspect of the piglet is as important as issues related to pre- and post-weaning management.

2.4 Positive impacts of alkaline phosphatase on piglet intestinal health

In current scientific literature, there are few studies conducted with the use of IAP in piglet diets, as well as the knowledge about its importance in the intestinal health of the host. In this introductory part, we discuss in general the main problems that the alkaline phosphatase enzyme can alleviate and report each of them.

LPS is characterized as the most abundant glycolipid complex present in the outer membrane of Gram-negative bacteria (i.e. *E. coli*). It is a complex molecule, negatively charged, composed of a polysaccharide chain called the O-specific chain and a lipid portion called lipid A. Lipid-A, present in the LPS molecule, expresses its endotoxic activity, and this fraction is responsible for stimulating the innate immune response of the host (AKIRA et al., 2001; BEUMER et al., 2003).

The pathogen recognition by the host immune system is mediated by pattern recognition receptors (PRR), which refer to molecular patterns associated with a wide group of pathogenic microorganisms. TLR function as PRR and play an important role in the host immune system (i.e. development of antigen-specific adaptive immunity), such as pathogen recognition, signaling and inflammatory response induction (WERLING & JUNGI, 2003; TAKEDA et al., 2003; TAKEDA & AKIRA, 2004). According to Takeda et al. (2003), the inflammatory response induced by TLRs is dependent on a common signaling pathway that is mediated by the adaptor molecule myeloid differentiation factor 88 (MyD88).

TLR4 recognizes LPS present in the outer membrane of Gram-negative bacteria, signaling the host immune response and TLR5 recognizes Gram-negative and Gram-positive bacterial flagella (ABASHT et al., 2008). This recognition of TLR4 occurs through macrophage recruitment (BEUTLER & RIETSCHEL, 2003) and subsequent mast cell degranulation with release of inflammatory mediators and proteins that stimulate the immune system through the release of tumor necrosis factor (TNF-α) by macrophages, nuclear factor kappa B (NF-κB) and cyclooxygenases (COX-2), besides the action of neutrophil at infection sites of infection (BEUMER et al., 2003; BATES et al., 2007; MUSSÁ et al., 2013).

The alkaline phosphatase enzyme has the ability to attenuate the LPS-mediated

inflammatory response, probably by dephosphorylation of the lipid-A portion present in LPS (CHEN et al., 2011). Beumer et al. (2003) reported a difference in TNF-α release among pigs that were treated with LPS or LPS + IAP. Also, in this study, the authors observed that 80% of the mice tested survived the lethal infection of Gram-negative bacteria when treated with the IAP isoenzyme, suggesting that the enzyme's action reduces the induction of the inflammatory response. Several studies have shown that IAP activity (KOYAMA et al., 2002; BATES et al., 2007; GOLDBERG et al., 2008) and the expression of TLR4 and IAP (ABASHT et al., 2008) increase in the presence of LPS, data that were also recently presented in studies developed by Melo et al. (2016).

However, LPS is not the only bacterial structure capable of inducing inflammatory response and is not the only target of IAP (CHEN et al., 2010). Bacterial structures such as CpG-DNA and flagella are recognized in the host by TLR9 and TLR5 receptors, respectively and, as well as LPS, recognized by TLR4, can cause inflammatory response and induction of cytokine production (SHINKAI et al., 2006; TOHNO et al., 2006). IAP's actions in dephosphorylation CpG-DNA, bacterial flagella and LPS inhibit the induction of proinflammatory cytokine IL-8 mediated by the recognition of these bacterial structures by the host (CHEN et al., 2010).

In addition, IAP also has the ability to dephosphorylate the nucleotide uridine diphosphate (UDP), a proinflammatory involved in inflammatory bowel disease (IBD) released by the host during inflammatory processes (MOSS et al., 2013). The aforementioned authors also reported that UDP release increases the expression of $P2Y_6$ receptors, which have been presented as stimulants of IL-8 production. In the same way that IAP dephosphorylates UDP, the enzyme also promotes the growth of commensal organisms by dephosphorylation the adenosine triphosphate (ATP) nucleotide. The presence of bowel luminal ATP inhibits the growth of commensal bacteria; however, when dephosphorylated in adenosine diphosphate (ADP) and adenosine monophosphate (AMP), the inhibitory effect of bacterial growth was not observed (MALO et al., 2014).

IAP has the ability to recompose the gut commensal microbiota in dysbiosis situations, which are often factors related to early-weaned piglets, therapeutic treatment with AGP and the causes of bowel disease promoted by increased susceptibility of the host to opportunistic enteric pathogens such as *Salmonella enterica* serovar Typhimurium and *Clostridium difficile* (ALAM et al., 2014). In the same study, the authors reported that the use of oral IAP promoted a decrease in inflammation in the colon, as evidenced by histology and blunted IL-1β response.

IAP’s function in cleaving ATP may also promote the maintenance of bowel

homeostasis due to the effect of luminal ATP in stimulating the release of bicarbonate (HCO_3^-) by the enterocyte (MIZUMORI et al., 2009). In the absence of IAP, the HCO_3^- secretion increases with ATP involvement via P2Y receptor activation (AKIBA et al., 2007). However, in the presence of IAP, the accumulated ATP in the bowel lumen is dephosphorylated, regulating HCO_3^- secretion. Thus, the IAP presents protective function of the bowel mucosa by mediating HCO_3^- secretion, maintaining homeostasis and preventing cell injury due to local acidification, since bowel pH influences enzyme activity and expression.

IAP activity was found at pH 8 to 10 (KOYAMA et al., 2002) and pH 7.5 (POELSTRA et al., 1997), while at pH 5 (POELSTRA et al., 1997) and 2.2 (AKIBA et al., 2007) showed no enzymatic activity. Corroborating this idea, Akiba et al. (2007) reported that duodenal secretion of HCO_3^- alkalizes the microclimate surrounding IAP, increasing its activity, and found that L-cysteine inhibited IAP activity *in vitro* and *in vivo* through the production of hydrogen sulfide, and increased acid-induced duodenal HCO_3^- secretion *in vivo*, confirming its effectiveness as an IAP inhibitor. Also, in this study, L-phenylalanine inhibited less *in vitro* IAP activity and partially increased acid-induced duodenal HCO_3^- secretion, while the D-phenylalanine had no effect on the *in vitro* IAP activity and *in vivo* HCO_3^- secretion.

Another effect of IAP is the minute-by-minute regulation of calcium (Ca^{2+}) absorption through bowel pH modulation, in which at alkaline pH its activity reduced bowel pH due to the enzyme concentration and luminal Ca^{2+} content, and at low pH, the Ca^{2+} absorption was reduced (BRUN et al., 2014). This IAP effect on reducing pH reveals that a part of Ca^{2+} absorption is independent of the vitamin D action and thus IAP acts by modulating the absorption of high Ca^{2+} concentrations in the enterocyte, which could promote a potential toxic effect. In addition, Lowe & John (2018) reported that zinc and magnesium minerals are important IAP cofactors, that is, IAP activity and the induction of metabolic processes by IAP has a direct and indirect relationship with some minerals.

Studies have also been carried out on the potential therapeutic effect of IAP on IBD in humans using rats as an experimental model (TUIN et al., 2009; MARTÍNEZ-MOYA et al., 2012). Treatment of colitis with alkaline phosphatase in rats resulted in lower colon weight and macroscopic damage scores, besides to normalizing neutrophil marker expression (S100A8, LCN2 and IL-1β) (MARTÍNEZ-MOYA et al., 2012). In addition, rats with colitis treated with alkaline phosphatase administered orally or rectally had lower bacterial translocation when compared to rats in the negative control and to rats that received antibiotics (MARTÍNEZ-MOYA et al., 2012). These data have demonstrated the beneficial effect of alkaline phosphatase in reducing the inflammatory response and risk of sepsis, which are evident

when IAP activity is reduced by weaning (LACKEYRAM et al., 2010) or by a specific inhibitor (MARTÍNEZ-MOYA et al., 2012).

Other studies indicate that oral administration of IAP has beneficial effects in situations of severe intestinal epithelial injury, while in moderate inflammation endogenous IAP may be sufficient to counteract the aggravating effects of LPS. In addition, an approach that includes treatment with IAP has a therapeutic promise in case of severe IBD (BOL-SCHOENMAKERS et al., 2010).

Corroborating these reports, Chen et al. (2011) mentioned that IAP is a defense factor of the bowel mucosa, a local immunomodulator, perhaps regulating the interaction of the LPS-TLR4 receptor between the commensal microbiota and the bowel epithelium. Finally, the IAP gene family has a strong evolutionary link with changes in GIT anatomy and food-induced microbial composition (LALLÈS, 2014).

However, alkaline phosphatases (ALP) are a group of isoenzymes present in different body tissues and with distinct physicochemical properties. According to Lowe & John (2018), ALP are located on the outer layer of the cell membrane in order to catalyze the hydrolysis of organic phosphate esters present in the extracellular space. In the liver, ALP is cytosolic and present in the hepatocyte canalicular membrane. ALP is present in decreasing concentrations in the placenta, ileal mucosa, kidneys, bones and liver, but the majority of IAP in the serum (more than 80%) is released from the liver and bone and in small amounts from the bowel, presenting high expression in the intestinal villi (SUSSMAN et al., 1989).

In recent reviews, Fawley & Gourlay (2016) and Haarhaus et al. (2017) reported that there are four ALP isoenzymes (Table 2).

Table 2. Description of alkaline phosphatase isoenzymes and isoforms.

Gene	Protein	Isoforms	Localization	Functions
ALPL	ALP, tissue-nonspecific isozyme (TNALP)	Bone-specific TNALP (B/l, B1, B1x and B2)	Skeletal tissue, kidney, neutrophil granulocytes, developing nervous system, and other cell types (vascular cells)	(1) Involved in skeletal mineralization, (2) vitamin B6 metabolism and (3) hypophosphatemia (genetic absence)
		Liver-specific TNALP (L1, L2 and L3)	Liver	Unknown so far
ALPI	ALP, intestinal-type (IAP)	Unknown so far	Mainly in duodenum	(1) Involved in fat absorption, (2) detoxification of LPS and free nucleotides, and (3) regulation of intestinal microbiota
ALPP	ALP, placental-type (PALP)	Unknown so far	Syncytiotrophoblasts, several tumours	(1) Tumor marker and (2) detoxification of bacterial toxins
ALPPL2	ALP, placental-like (PLALP)	Unknown so far	Testis, malignant trophoblasts, testicular cancer	Unknown so far

Source: Adapted from Fawley and Gourlay (2016); Haarhaus et al. (2017).

In short, deleterious effects on the bowel can be attributed to the reduction of IAP expression and its potential protective effect on post-weaning piglets, with diet being a factor capable of interfering with the modulation of this enzyme (GOLDBERG et al., 2008). Thus, considering the physiological events that occur in piglets as a result of weaning, IAP can contribute positively to pig production when added via diet, due to the several benefits (Table 3).

Table 3. Properties of intestinal alkaline phosphatase enzyme.

General and specific functions	References
IAP secretion in the basolateral membrane	Lallès, 2014; Fawley and Gourlay, 2016; Haarhaus et al., 2017
Fatty acid absorption regulation	Mahmood et al., 2002; McConnell et al., 2009; Lallès, 2014; Fawley and Gourlay, 2016; Haarhaus et al., 2017
Dephosphorylation of ATP, ADP and AMP molecules	Mizumori et al., 2009; Lallès, 2014; Malo et al., 2014; Fawley and Gourlay, 2016; Haarhaus et al., 2017
Dephosphorylation of specific bacterial components including LPS, CpG DNA, Pam-3-Cys and flagellin	Chen et al., 2010; Moss et al., 2013; Lallès, 2014; Fawley and Gourlay, 2016; Hamarneh et al., 2017; Haarhaus et al., 2017
Modulation of duodenal bicarbonate secretion and enteric surface pH	Akiba et al., 2007; Mizumori et al., 2009; Brun et al., 2014; Lallès, 2014
Reduction of intestinal bacterial translocation	Chen et al, 2011; Lallès, 2014; Malo et al., 2014; Fawley and Gourlay, 2016; Haarhaus et al., 2017; Bilski et al., 2017
Intestinal tolerance and development of commensal bacteria	Lallès, 2010; Lallès, 2014; Malo et al., 2014; Bilski et al., 2017
Control of local and systemic inflammation or infection	Beumer et al., 2003; Tuin et al., 2009; Bol-Schoenmakers et al., 2010; Ramasamy et al., 2010; Martínez-Moya et al., 2012; Moss et al., 2013; Alam et al., 2014; Lallès, 2014; Fawley and Gourlay, 2016; Bilski et al., 2017; Hamarneh et al., 2017
Nucleotide dephosphorylation	Lallès, 2014; Fawley and Gourlay, 2016; Haarhaus et al., 2017
IAP secretion in the apical membrane (microvilli)	Lallès, 2014; Fawley and Gourlay, 2016; Haarhaus et al., 2017
Ca^{2+} absorption regulation	Brun et al., 2014; Lallès, 2014; Fawley and Gourlay, 2016; Haarhaus et al., 2017
Inorganic phosphate homeostasis	Sasaki et al., 2018

Within this context, IAP can act as a host defense factor (KOYAMA et al., 2002), but a better understanding of the factors that regulate the expression of IAP and the promotion of intestinal health is necessary, with reduction of inflammatory processes, which may contribute to the treatment and prevention of IBD and, consequently, improve the health status and growth performance of animals.

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BIOLOGICAL RESPONSE OF PIGLETS CHALLENGED WITH ESCHERICHIA COLI K88 WHEN FED DIETS CONTAINING INTESTINAL ALKALINE PHOSPHATASE

ABSTRACT - The aim of this article was to test the effect of intestinal alkaline phosphatase (IAP) added in diets on growth performance, diarrhea occurrence (DO), blood metabolites, relative organs weight and intestinal morphometry of weaned piglets challenged with enterotoxigenic *Escherichia coli* (ETEC) K88^{+}. A total of 64 crossbred entire male piglets, with an average initial body weight of 7.168 ± 0.287 kg were allocated into four treatments: control diet (CD^{-}), CD^{-} + antimicrobial growth promoter (AGP), CD^{-} + 15 mg IAP/kg of diet or CD^{-} + 30 mg IAP/kg of diet. All piglets were orally challenged with 6 mL of 10^{6} CFU/mL. In pre-starter I phase, piglets that received 30 mg IAP added in the diet or CD^{-} showed better feed conversion rate (P = 0.075) compared to those fed 15 mg IAP. Piglets that consumed 30 mg IAP or CD^{-} showed greater (P = 0.004) average daily body weight gain (ADBWG) in the pre-starter II phase. Piglets fed 15 mg IAP had lower average daily feed intake (ADFI) (P = 0.033) compared to piglets with diets containing AGP. At the total period, there was a difference between treatment, in which the piglets fed 15 mg IAP showed a reduction in ADBWG (P = 0.040) and ADFI (P = 0.092). For the pre-starter II phase, there was a difference (P = 0.044) of treatment, in which the piglets that consumed the diet containing 30 mg IAP showed a 24% improvement in DO compared to the treatment with 15 mg IAP. We observed the main effect (P = 0.009), with the addition of 30 mg IAP in the post-challenge phase in decreasing piglet DO (5.56%) when compared to those receiving AGP (16.67%). For the total period, piglets that consumed 15 mg IAP showed greater (P = 0.007) DO when compared to those receiving 30 mg IAP. No differences between treatments were obtained in any of the pre- and post-challenge plasma concentration indicators. The spleen relative weight of piglet increased (P = 0.043) in response to 30 mg IAP treatment. Based on the present results, the addition of 30 mg IAP in diets improves the growth performance and attenuates the DO in piglets. In addition, the results suggest that 30 mg IAP promotes an increase in spleen relative weight to maintain intestinal health status.

Keywords: alkaline phosphatase, antimicrobial growth promoter, blood metabolites, feed additives, intestinal morphometry, post-weaning diarrhea.

1 INTRODUCTION

The development of the gastrointestinal tract (GIT) of piglet in the post-weaning is a highly specialized, dynamic and constantly changing process such as organs size, protein turnover rates, microbiota composition, rapid and marked changes in digestive, absorptive, physical and/or selective barrier, and immune functions (PLUSKE, 2016; MOESER et al., 2017). In this sense, a clear definition of effective gastrointestinal functionality and how it can be measured is necessary to monitor animal health and assess the effects of any nutritional intervention on growth performance (CELI et al., 2017). In overall terms, "intestinal health" encompasses a series of physiological and functional variables (PLUSKE et al., 2018), a healthy microecosystem (LIAO et al., 2017) and is closely associated with growth performance analysis and economic values (SUN & KIM, 2017).

An optimally functioning GIT is clearly important for overall metabolism, physiology, architecture of intestinal structures and cells, disease status and growth performance of weaned piglets (PLUSKE et al., 2018). For this, basic and handling measures are indispensable in this transition phase, which include appropriate sanitation conditions, animal welfare, practices related to feeding and nutrition, and piglet agglomeration rate (JAYARAMAN & NYACHOTI, 2017). Besides that, the complex interactions that occur in the GIT between nutrition, mucosa and intestinal microbiota, and the enteric nervous system (ADEWOLE et al., 2016; MOESER et al., 2017; LIU et al., 2018).

Recently, there is a wide range of products such as feed additives (organic and inorganic acids, high levels of zinc oxide, essential oils, herbs and spices, some types of prebiotics, bacteriophages, antimicrobial peptides), feeding/nutritional strategies, nutraceuticals/functional foods and management practices that influence or intend to influence different aspects of intestinal health and mitigate negative effects from abrupt weaning (ADEWOLE et al., 2016; JAYARAMAN & NYACHOTI, 2017). All these factors have been studied because they directly and/or indirectly influence the functionality of the GIT.

On the other hand, any discussion on intestinal health in the post-weaning period should include the potential impacts of enterotoxigenic *Escherichia coli* (ETEC) strains (YI et al., 2016; GRESSE et al., 2017), mainly including F4 (K88)$^+$ and F18$^+$ (SUN & KIM, 2017). ETEC is capable of synthesizing a wide range of enterotoxins and thermostable peptide toxins (ABUBAKAR et al., 2017). Thus, it is necessary to search for new safe and effective solutions to minimize losses caused by bacterial infections, since the use of long-term antibiotics and

therapeutic doses of antimicrobials can contribute to the reduction of bacterial diversity and increase of inflammatory bowel disease.

Studies evaluating the use of intestinal alkaline phosphatase (IAP) isoform in intestinal health have been reported and include experiments on the effects of IAP on specific bacteria and bacterial components (CHEN et al., 2010), the reduction of inflammatory activity of TNF-α levels (MOSS et al., 2013), protection against diarrhea and other enteric infections (ALAM et al., 2014) and the ability to promote bacterial growth and normalize the intestinal microbiome (MALO et al., 2014).

Our hypothesis was that the addition of IAP in diets would improve the intestinal health status and consequently promote a benefit on the growth performance of piglets in the nursery phases, being an alternative to traditional antibiotics used as performance enhancers. Thus, the aim of this study was to test the effect of intestinal alkaline phosphatase added to diets on the growth performance, diarrhea occurrence, blood metabolites, intestinal morphometry, the relative organ weight of weaned piglet challenged with *Escherichia coli* K88^{+}, and the *in vitro* simulation of microencapsulated IAP on the pH modulation capacity and its dilution in acidic and basic solution.

2 MATERIAL AND METHODS

The experiment was carried out in the Swine Sector of the Experimental Farm Professor Antonio Carlos dos Santos Pessoa of the State University of Western Paraná - UNIOESTE, Campus of Marechal Cândido Rondon/Paraná, Brazil. The piglets were carefully managed to avoid unnecessary discomfort and all experimental procedures were approved by the UNIOESTE Research Ethics Committee (No. 13/19 - CEUA).

All procedures of euthanasia for the animals were performed by electronarcosis, in compliance with the Normative Resolution No. 37 of February 15, 2018 of CONCEA-Brazil, which establishes the Guidelines of the Practice of Euthanasia of the National Council for Control of Animal Experimentation.

2.1. Experimental design, animals, housing and diets

A total of 64 crossbred piglets (Landrace x Large White), entire male weaned at 25-days-old with an average initial body weight of 7.168 ± 0.287 kg were assigned in a randomized complete block design consisting of four treatments repeated twice in the time, totaling 32 experimental units (EU), with two animals per EU.

At the beginning of the experimental period, the animals were weighed and identified with numbered ear tags and housed in a masonry nursery shed and ceramic roof tiles, consisting of suspended pens (1.545 m²), with polyethylene plastic flooring, equipped with nipple-type drinking fountains and gutter-type feeders, arranged in two rows, divided by a central corridor, where they remained for a period of 19 d.

The ambient temperature and relative humidity were recorded using a data logger with digital display (Vketech brand, temperature instruments model), which was installed in the center of the experimental building. The minimum recorded temperature of the internal environment was 19.1 ± 5.2ºC and the maximum was 29.7 ± 5.5ºC. The nursery shed 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 growth phases I and II, following the nutritional recommendations proposed by Rostagno et al. (2017). The experimental treatments (Table 1) were composed of a control diet (negative control), control diet + antimicrobial growth promoter (AGP, 150 mg tiamulin/kg of diet), control diet + 15 mg IAP/kg of diet and control diet + 30 mg IAP/kg of diet.

2.2. Sample collection, preparation and analytical procedure

2.2.1. Process of IAP microencapsulation

The methodology used for 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. After the dilution step, the temperature of the mixer was reduced because there was information on the possibility of inactivating the enzyme with the use of high temperatures. The first PGE emulsifier was added with the mixer at a temperature of approximately 5°C, in which it was chosen because it showed a greater tendency to solidify in contact with the product, in this case, when added the first agglomerates were formed. After further cooling the PA was added, which gave greater structure and finished the agglomeration process and protection of the enzyme. Because it is a process of low production, an industrial stirrer was used and the PGE was added at 55°C in the first step and the PA at 65°C taking into consideration the melting points of each component. As seen on day, the enzyme in contact with the water remained intact showing that there was no RS available for this contact. The final composition of the vehicle used in the microencapsulation process presented the following proportions: 50% RS, 30% PGE + 20% PA.

2.2.2. Process of evaluation of post-microencapsulation IAP activity

IAP was microencapsulated in 50% RS + 50% propylene glycol ester (IAP I) or microencapsulated in 50% RS + 50% propylene glycol ester with palmitic acid (IAP II). After the microencapsulation process, the IAP activity on adhesion and the phagocytic activity of the equine bronchoalveolar macrophage was evaluated. The microencapsulated enzymes were properly weighed and diluted in 50% ethanol for 24 h, kept under stirring in an oven at 37°C.

The procedures tested were IAP in the forms: pure lyophilized (IAP), IAP I and IAP II induced or not with *E. coli* LPS. As control procedures were used the alveolar macrophage (AM), AM + phosphate-buffered saline (PBS) and AM + IAP groups, without the addition of LPS (microcytic activity inducer). Macrophages were isolated from bronchoalveolar lavage (BAL) from a healthy mare with 360 kg of body weight. The animal was fasted for 6 h prior to collection to perform sedation using intravenous (IV) detomidine (Eqdomin, 0.02 mg/kg, Ourofino, São Paulo, Brazil) and pethidine chloride IV (Dolosal, Cristália, São Paulo Brazil). BAL fluid collection was performed with a flexible silicone catheter of 300 cm length and 8

mm diameter (V-PBAL-300, Cook Vet Products, Hamburg, Germany). It was introduced via the nasotracheal tube until it was lodged in a bronchus, when the cuff at the distal end was inflated with 10 mL of air. An amount of 500 mL of sterile saline solution (0.9% NaCl) preheated to 37°C was used (HOFFMAN, 2008), infused into 125 mL aliquots and aspirated after each aliquot. The collected material was stored on ice in tubes until it reached at the laboratory.

In the laboratory, BAL fluid was centrifuged at 340 g for six minutes at 4°C (MICHELOTTO et al, 2010) and the pellet obtained was suspended in the animal's own supernatant. Total nucleated cell count was performed in the Neubauer chamber, in two opposite quadrants, and cell viability was verified through trypan blue. From the cell pellet, 10 µL was used for slides mounting, which were stained with the Romanowski technique (Panotic Rapid, Laborclin, Paraná, Brazil) for differential cell count in 1000x magnification. Two 2 mL aliquots of BAL fluid supernatant were separated in the centrifugation phase and frozen at -20°C for further analysis.

After counting the total number of cells obtained in the BAL fluid, the cell suspension was adjusted to the concentration of 5 x 10^6 viable cells/mL. An amount of 100 µL of the adjusted suspension was plated into 96-well microplate wells (in triplicate) to analyze adhesion and phagocytic activity (one plate for each assay). The plates were incubated for 1 h at 37°C. After this period, the supernatant was discarded, leaving only the AM adhered to the plastic. After the isolation of the AM, the phosphate-saline buffer (PBS, control group) and ETEC lipopolysaccharide (LPS, 100 ng/mL) inflammatory inducers were added in the amount of 50 µL. The plates were incubated for an additional 60 minutes in an oven at 37°C, then the treatments were carried out: T1: PBS; T2: AM + PBS; T3: AM + IAP; T4: AM + LPS; T5: AM + LPS + IAP; T6: AM + IAP I; T7: AM + LPS + IAP I; T8: AM + IAP II and T9: AM + LPS + IAP II.

After 1 h of incubation of the treatments, the supernatant containing the treatments on the plate was discarded, the wells were washed twice with PBS and the adherent cells were then fixed with 50% methanol for 10 minutes at ambient temperature. After this time, the supernatant was discarded and 100 µL of 0.2% Giemsa dye (Sigma-Aldrich, St. Louis, Missouri, United States) was added to each well for 40 minutes at ambient temperature. The wells were again washed twice with PBS and 200 µL of 50% methanol was added. After 30 minutes, the resulting solution had its absorbance read in a spectrophotometer with a wavelength corresponding to 550 nm (Sunrise, Tecan, Switzerland). This test was adapted from the technique of Rosen &

Gordon (1987). The final adhesion result was corrected by the percentage of alveolar macrophages in the BAL fluid (absorbance x % macrophages).

To analyze the phagocytic activity of the alveolar macrophage, after the macrophage plates had their wells washed twice with PBS, as in the previously mentioned assay, 100 µL PBS and 20 µL zymosan (Sigma-Aldrich, St. Louis, Missouri, United States) were added to each well, stained with neutral red (6.7 mg/mL, Sigma-Aldrich, St. Louis, Missouri, United States) and incubated at 37°C for 30 minutes. Then, the supernatant was discarded and the cells were fixed with Baker's solution (4% formaldehyde, 2% sodium chloride, 1% calcium acetate, aqueous solution) for 30 minutes at 37°C. The supernatant was discarded and 100 µL PBS was added to perform centrifugation at 400 g for 5 minutes. Then, after discarding the supernatant, the neutral red dye was solubilized with 200 µL extraction solution (10% glacial acetic acid, 40% ethanol in aqueous solution) and incubated for 30 minutes. After this period, the absorbance was measured at 550 nm. This assay was adapted from the method described by Dyrynda et al. (1998). The final result of phagocytic activity was corrected by the percentage of alveolar macrophages in the BAL fluid (absorbance x % macrophages).

2.2.3. Solubility and pH change of post-microencapsulation IAP

In vitro solubility was determined by the percentage of microencapsulated IAP weight loss and pH change with the aid of a digital pH meter (Hanna Instruments Inc., Rhodes Island, USA, model HI 99163). A sample amount of 15.0005 ± 0.0002 g was weighed into identified and pre-weighed Erlenmeyer flask (n = 8). Subsequently, 100 mL of an acidic solution (0.1 mol HCl in distilled water) with an initial average pH of 1.41 or a basic solution (0.5% sodium dodecyl sulfate in distilled water) with an initial average pH of 7.2 in each flask (in quadruplicates) were added, sealed with aluminum foil, allowed to stand for 5 minutes and then placed in a water bath at 37°C for 24 h. Before sealing the flasks with aluminum foil, the initial pH of each solution was measured in order to simulate different pH media. At each time interval (0.5, 1, 17 and 24 h), pH measurement and agitation of the flasks was performed for 1 minute. After 24 h, the solution with the microencapsulated IAP was filtered through pre-weighed quantitative filter paper (Unifil Brand, C41, 125 mm diameter, 0.2 mm thickness) washing the remainder with distilled water. The retained in the filter and flask was dried for 24 h in a ventilated oven at 65°C. Another four flasks containing the acidic and basic solution were filtered and rinsed in the same way ("blank"). Such blanks were made and the average weight

decrease of the four filter papers after drying was used to adjust the weight of dried sample. Solubility was expressed as a percentage of weight loss.

2.3. Growth performance testing and diarrhea occurrence (DO)

The animals received feed and water *ad libitum* throughout the experimental period. Performance variables were determined on days 0-10, 10-19, 15-19 and 0-19 of the experiment, through the body weight (BW) of the animals and the quantification of the feed provided and wasted on the floor and feeder of each pen. BW was recorded (stainless steel digital scale, model UL50i) at the beginning and end of each experimental phase. BW were: 7.168 to 8.893 kg for pre-starter I (25 to 35 d of age), 8.893 to 11.191 kg for pre-starter II (35 to 44 d of age) and 9.428 to 11.191 kg for challenge (40 to 44 d of age). Based on these data, the average daily feed intake (ADFI, kg/day), average daily body weight gain (ADBWG, kg/day) and feed conversion rate (FCR, kg/kg) were calculated. The DO was recorded daily, in the morning at 9 am, priori to cleaning the experimental unit. Presence or absence of diarrhea (liquid feces on the floor and/or dirty anal region) was calculated as the proportion of days with diarrhea occurrence.

2.4. Blood sampling

In order to evaluate the blood metabolite concentration before (baseline) and after the beginning of the experimental treatments, piglets were kept on an 8-h fasting diet at the end of each experimental phase. Then, blood collection (± 10 mL) was performed via puncture of the anterior cranial vena cava of 32 animals using 0.7 x 30 mm gauge needles. After the blood was transferred to two glass tubes, one containing heparin and one with sodium fluoride, which were labelled, stored in a Styrofoam box with ice and sent to the Blood Parameters Laboratory. The blood samples were centrifuged (Centrilab Model 80-2B analog centrifuge) at 3,000 *g* for 10 minutes. Approximately three mL of each tube were then transferred to previously identified and frozen “Eppendorf” polyethylene tubes for analysis of urea (enzymatic-colorimetric method), glucose (enzymatic-colorimetric method) and alkaline phosphatase (kinetic-colorimetric method). These analyses were determined by spectrophotometry (Bel SPECTRO S05) using specific Gold Analytical Diagnostic kits. To calculate the blood metabolite values of the total period, the concentration values obtained in each phase during the experiment were considered.

2.5. Bacterial strain and challenge procedure

Four days before slaughter, the 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 of ETEC K88$^+$ provided by the Laboratory of the Mercolab (Cascavel, PR, Brazil). ETEC was multiplied in brain heart infusion broth for 18-24 h at 36 ± 1°C until reaching the concentration of 1.0 x 10^9 CFU/mL. Subsequently, serial dilution was performed in saline solution (0.9% NaCl) up to the concentration of 1.0 x 10^6 CFU/mL. The infection via in piglets was at the back of the oral cavity using a syringe. The rich solution in ETEC was slowly dripped into the piglet's throat so that the swallowing reflex was triggered and the inoculant's passage into the lungs was minimized (OWUSU-ASIEDU et al., 2003).

2.6. Samples of the intestinal epithelium

On the 19th days of experimentation, six animals from each experimental treatment were slaughtered (after a 6-h fasting) following humane slaughter methods (electronarcosis followed by exsanguination) for data collection and biological samples for intestinal epithelial morphometric analysis. The choice of animal to be slaughtered was according to body weight, being the one that presented the body weight nearest to the average treatment. Fasting was performed to reduce the presence of residues in the organs, facilitate their handling and avoid damage to tissues that were used for morphometric analysis.

To evaluate the structures of the intestinal epithelium, immediately after organ removal, segments of approximately 3-cm length of the jejunum (extracted at 150 cm from the ileocecal junction) (GUO et al., 2001) were collected, washed with physiological solution (0.9% NaCl) and stored in 50 mL sterile plastic pots 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 Histopathology Laboratory of the Mercolab (Cascavel, PR, Brazil) where they were paraffin-embedded and microtomized for slides mounting. The paraffin blocks containing the samples were cut in a microtome (ANCAP 78), sections were performed and transferred to the slides.

The slides were stained with hematoxylin and eosin for histological description (GAO et al., 2000). The analyses were evaluated with the aid of an optical microscope and the computer program ZEN 2.0 Image software. Ten villus measurements and respective crypts were analyzed per sample. The villi were measured from the extremity nearest to the intestinal lumen

to the beginning of the crypt. The crypt was measured from the end of the villus to the end of the crypt cell layer.

2.7. Relative organ weight (%)

The digestive organs (empty stomach, liver + gallbladder, empty small bowel + pancreas, empty cecum and colon) and non-digestive organs (spleen and kidneys) were removed, washed with water and weighed (digital electronic balance, model 9094, Toledo brand), and the length of the animals' small bowel was measured. With the data, the relative organ weight (%) was calculated, considering the animals' body weight at the time of slaughter.

2.8. Calculations and statistical analyses

Before evaluating the result of covariance (ANCOVA) and variance (ANOVA) analysis, the standardized residuals analysis of Student (RStudent) was performed in order to identify influential observations or outliers. The criterion adopted for identification of outliers was based on a normal distribution curve; RStudent values greater than or equal to three standard deviations were considered as influential. The normality of experimental errors and the homogeneity of error variances between treatments for the several variables were previously evaluated using the Shapiro-Wilk and Levene tests, respectively.

For the characteristics of growth performance, blood metabolites and relative organ weight the statistical model used was: $Y_{ijk} = m + T_i + b_j + \beta (X_{ijk} - \bar{X}_{...}) + \varepsilon_{ijk}$. The effects of the factors included in the model were described by: 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; m = 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); β = regression coefficient of Y over X; X_{ijk} = average observation of the covariate (initial body weight, initial blood metabolite, weight of digestive and non-digestive organs using the slaughter weight of animals as covariate) in each plot, measured in the i-th treatment class, at the j-th block and in the k-th replication; $\bar{X}_{...}$ = overall average for the covariate X; ε_{ijk} = random error of the plot associated with level i, block j and replication k. For the characteristics of intestinal morphometry, the statistical model used was the one previously mentioned, without including the covariate effect.

For the statistical analysis of the diarrhea occurrence (DO), the data were transformed into binary values, wherein: 0 = diarrhea absence and 1 = diarrhea presence, being presented as percentage results (%). For the DO variable, the "Generalized Linear Model" (GLM) was fitted to the data using the binomial distribution and logit link function. GLM 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 together with the graphical analysis of residue adherence. The DO was compared using a test of the difference between the lsmeans, through the χ^2 statistic.

The effects of the experimental treatment classes on the dependent variables (growth performance and blood metabolites) were verified through ANCOVA. Comparisons between treatment averages were performed according to Tukey's post-hoc test. The β error of the growth performance parameters was used to help explain the probability values between 5% and 10% of significance. This procedure was performed using the test power of the package power.anova.test (R Core Team, 2017). The other variables were analyzed using the "General Linear Models" procedures of the statistical software "SAS University Edition" (SAS Inst. Inc., Cary, NC, USA). All data are presented as averages with pooled standard error of the mean.

3 RESULTS

Experimental errors of the growth performance, blood metabolites, intestinal morphometry and relative organ weight variables presented normal probability distribution and treatment variance homogeneity ($P > 0.05$). In all phases, there was an effect ($P < 0.05$) of initial body weight (IBW) covariates on the growth performance variables and baseline alkaline phosphatase (ALP) on final ALP, indicating the need to correct the observed averages of these characteristics. There was no effect ($P > 0.05$) of IBW covariates on FCR, glucose and initial urea on glucose and final urea.

3.1. Process of evaluation of post-microencapsulation IAP activity and solubility and pH change

It was observed that macrophage adhesion was higher for treatments composed of AM + LPS + IAP I and AM + LPS + IAP II. However, the phagocytic activity related to macrophage adhesion was lower for the groups AM + LPS + IAP, AM + LPS + IAP I and AM + LPS + IAP II compared to the other treatments. The group with AM + LPS + IAP II presented the lowest phagocytic activity. In short, pure or microencapsulated IAP showed activity even after handling and microencapsulation process, respectively. After the pilot analysis of the enzymatic activity, the IAP II microencapsulation method was selected to continue processing the enzyme and its addition to the diet.

When the capacity of the microencapsulated IAP was verified in modulating the pH or to show its activity (Figure 1), a pH reduction in the acidic solution + IAP was obtained at different times and a reduction followed by a slight increase of the pH in the basic solution + IAP.

3.2. Growth performance testing and diarrhea occurrence (DO)

Prior to the growth performance test, none of the animals showed clinical signs of post-weaning diarrhea. IAP has the ability to improve the growth performance of piglets in the pre-starter II phase and total period when used at a dietary dose of 30 mg/kg (Table 2). First, in order to assess the role of IAP in a relevant way, pre-challenge and post-challenge performance variables were determined. Before the challenge with ETEC, in pre-starter I phase, piglets that received 30 mg of IAP added in the diet or control group showed better

feed conversion rate (P = 0.075) compared to those fed 15 mg of IAP. Piglets that consumed 30 mg of IAP or control group showed greater (P = 0.004) average daily body weight gain (ADBWG) in the pre-starter II phase. Piglets fed 15 mg of IAP had lower average daily feed intake (ADFI) (P = 0.033) compared to piglets with diets containing AGP. When we evaluated the effect of IAP over the total period, there was a difference between treatment, in which the piglets fed 15 mg of IAP showed a reduction in ADBWG (P = 0.040) and ADFI (P = 0.092) (Table 2).

The ability of IAP in piglet diets to reduce the DO was verified pre-challenge and post-challenge. In pre-starter I phase, the average DO reduction presented by piglets fed 30 mg of IAP was 13.85% when compared to those that received 15 mg of 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% improvement in DO compared to the 15 mg of IAP treatment. The main effect (P = 0.009) of DO reduction was with the addition of 30 mg of IAP in the post-challenge phase of piglets when compared to 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 DO when compared to those receiving 15 mg of IAP (Table 3).

3.3. Blood metabolites

No differences between treatments were obtained in any of the pre- and post-challenge plasma concentration indicators (Table 4). The values found are close to the established range of 13 to 45 mg/dL for urea and 65 to 120 mg/dL for glucose.

3.4. Samples of the intestinal epithelium and relative organ weight (%)

There was no effect (P > 0.05) of treatments on the intestinal morphometry of piglets (Table 5). Piglets that received treatment with 30 mg of IAP had greater (P = 0.043) relative weight (percentage of body weight) of the spleen when compared to control treatment (Table 6).

4 DISCUSSION

4.1. Process of evaluation of post-microencapsulation IAP activity and solubility and pH change

By reducing LPS-induced phagocytic activity, it is suggested that IAP reduced the toxic effect of LPS, since the TLR4 expression (not determined in this study) and IAP expression increase in the presence of LPS (ABASHT et al., 2008; CHEN et al., 2010). This result is associated with the IAP's ability to attenuate the LPS-mediated inflammatory response, hypothetically through dephosphorylation of the lipid A present in the LPS (CHEN et al., 2011).

The solubility of microencapsulated IAP was, on average, 3.51 times greater in the acidic solution when compared to the basic solution, which can be attributed to the final proportions and polarity of the compounds used as a vehicle in the microencapsulation process. In relation to pH oscillation, these results may be related to the absence of buffers in the solutions, wherein influenced the pH variations, and not due to the effect of IAP's ability to modulate the pH because the solubility was greater in the acidic solution and under these medium conditions there is no IAP activity or pH modulation capacity (POELSTRA et al., 1997; KOYAMA et al., 2002; AKIBA et al. 2007). In previous studies, it was found that IAP has the ability to modulate intestinal pH, in which in alkaline pH its activity reduced intestinal pH due to the enzyme concentration (BRUN et al., 2014).

4.2. Growth performance testing and diarrhea occurrence (DO)

In this study, the growth performance of piglets was influenced by dietary treatments. The lack of growth response attributable to AGP was unexpected, since for several decades it was one of the main feeding alternatives in the control of the post-weaning diarrhea. Alam et al. (2014) attributed antibiotics as an additive that inherently causes dysbiosis, an imbalance in the number and composition of intestinal commensal bacteria, which eventually did not occur in piglets in the control group or those that consumed 30 mg of IAP.

These results may be attributed by the explanations that IAP added in 30 mg acted as a host defense factor (KOYAMA et al., 2002), but a better understanding of the factors that regulate IAP expression and its effects on the promotion of intestinal health is needed, consequently, improves on the growth performance of piglets. In this first trial with piglets, it

was found that the addition of 15 mg of IAP in the diet was not sufficient to attenuate the critical period of post-weaning.

Based on previous experimental reports (MOSS et al., 2013; ALAM et al. 2014; LALLÈS, 2014; MALO et al., 2014), IAP is a defense factor of the intestinal mucosa, a local immunomodulator (CHEN et al., 2011), which positively influences the piglet's growth performance, demonstrating the favorable effect of IAP in reducing the intestinal inflammatory response (LACKEYRAM et al., 2010). However, the piglets in the control group also showed a favorable response, which may be related to the fact that the diet without additive was able to promote intestinal health status, adequately stimulate local defensive responses and beneficially influence the resident gastrointestinal microbiota (ADEOWLE, 2016) without affecting the growth performance variables. The reason for this inconsistency is that the efficiency of each additive depends on the diet (composition, feed processing, feeding methods), colonization and associated succession of microbial populations, stress and genetics (CELI et al., 2017).

The results of the present study for piglets that consumed 30 mg of 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 diarrhea occurrence and enteric infections because the mice maintained their body weight. Recent publications with the use of feed additives also found an improvement in growth performance when compared to the use of diets containing AGP (JIANG et al., 2015; MA et al., 2015; PEARCE et al., 2015; TRAN et al., 2016; WAITITU et al., 2016; VAN DER AAR et al., 2017; WU et al., 2017). However, the variation that occurs in the responses of piglets that consumed feed additives is presumably a consequence, in part, of the many different management conditions they are submitted (PLUSKE et al., 2018), amount added to the diet (ADEWOLE et al. , 2016) and differences in age, health status or environmental conditions (LIU et al., 2018).

IAP activity in the prevention of DO has been confirmed, since extensive epidemiological studies demonstrated that the antibiotic-associated diarrhea, 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 (ALAM et al., 2014). We speculate that the reason why piglets that consumed 15 mg of IAP showed no response to DO is supported by the amount that was added to the experimental diet, as its microencapsulation process is complex, leading to losses in its efficacy and lower enzyme activity in GIT.

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 AGP, which is demonstrated

by other studies conducted with mice (CHEN et al., 2011; MOSS et al., 2013; ALAM et al., 2014; MALO et al., 2014) and piglets (BEUMER et al., 2003). An impaired growth performance was not obtained in piglets challenged with ETEC, but they exhibited considerably a greater DO because ETEC infection is often associated with diarrhea and impaired intestinal barrier function (YANG et al., 2014). The F4 inoculation significantly increased the DO in piglets that did not consume 30 mg of IAP. These findings are attributed to the fact that IAP expressed the role of inhibiting adhesion and bacterial internalization, preventing disruption of barrier integrity and modulating cytokine expression (SONG et al., 2015), even though in our study we did not determine the cytokine concentration.

4.3. Blood metabolites

Notably, urea and glucose concentrations agree to the reference values for the species studied (COOPER et al., 2014; NIELSEN et al., 2015; WIJKSTROM et al., 2015; MANELL et al., 2016; PERRI et al., 2017). These findings are supported by the growth phase of piglets, in which the nutritional and physiological needs are altered, as well as protein levels in the feed, generating lower metabolite concentrations (PERRI et al., 2017). In addition, infectious diseases or the occurrence of an inflammatory process significantly reduce feed intake and cause nutrient redistribution from growth processes to support the immune system (OWUSU-ASIEDU et al., 2003).

Increased blood urea concentration also occurs due to high protein catabolism and stress. In this sense, it is possible that the treatment groups attenuated stress and the inflammatory process during the nursery phase because blood urea values are altered in situations of diseases and injuries (WILSON et al., 1972), age, gender, breed, nutrition and health status (KLEM et al., 2010; COOPER et al., 2014), and in such cases, amino acids are released from muscle degradation and can be used for the synthesis of acute phase proteins in the liver and as an energy source (OWUSU-ASIEDU et al., 2003).

Metabolic pathways for the synthesis of urea by the liver use energy and the increase in urea synthesis or plasma urea concentration can increase the energy expenditure by the liver and concomitantly reduce glucose that would be intended for other purposes. In addition, piglets that are subjected to challenged or show acute clinical signs of disease may result in a decrease in glucose concentration (WILSON et al., 1972) because they use much of their energy to maintain vital functions. However, the results obtained do not corroborate this idea and we attribute it to the fact that pigs are animals tolerant to glucose concentrations and

hyperinsulinemic (LANG et al., 1999; BELLINGER et al., 2006; NIELSEN et al., 2015).

Regarding plasma ALP concentrations, all treatment groups were analogous. However, piglets showed higher average plasma ALP values than those reported by Wang et al. (2012) and Zhang et al. (2018), who investigated the effect of zinc mineral on the ALP plasma concentrations of piglets.

As the plasma ALP concentration has a wide oscillation, being regulated by several factors such as diet (GUL et al., 2017; ZHANG et al., 2018), stress (MAYENGBAM & TOLENKHOMBA, 2015), body growth rate and age (WANG et al., 2012; ABENI et al., 2018), and genetics (KIEREK-JASZCZUK & GELDERMANN, 1985; ABENI et al., 2018; REYER et al., 2019), the results suggest that there is a physiological mechanism that activates the ALP activity in injuries, stress and pathological situations, and intestinal infections in piglets, such as a post-weaning and/or challenge period. The antibiotics addition in diets has as metabolic effect the increase in the IAP response. It is plausible that the AGP-fed piglets had higher average ALP values as a way to restore the intestinal microbiota (dysbiosis).

4.4. Samples of the intestinal epithelium and relative organ weight (%)

Intestinal histology was examined in several studies involving piglets and villus height (VH) reduction has been associated with post-weaning growth lag and reduction in ADFI (NYACHOTI et al., 2012; GAO et al., 2013; PAN et al., 2017; ANJOS et al., 2019). However, the dietary treatments did not affect the intestinal morphometry variables in the study, and there is no plausible explanation for these results, since the increase in OD and lower growth performance are related to reduced VH (RONG et al, 2015).

Shorter villi were also associated with the presence of serum toxins (not determined in the present study) (PAN et al., 2017). These shorter villi and lower VH:CD reflect the presence of fewer absorptive and more secretory cells in the small bowel and a greater amount of unabsorbed dietary material flowing to the large bowel, which would act as a substrate for ETEC and stimulate bacterial proliferation (GAO et al., 2013). On the other hand, the higher the VH and the lower the CD (higher VH:CD), the better will be the nutrient absorption capacity and the lower will be the energy losses with cell turnover (OWUSU-ASIEDU et al., 2003).

Intestinal damage as a result of ETEC infection was not observed in terms of intestinal morphometry in the present study. Piglets that received 30 mg of IAP showed a slight average increase (0.72) in the VH:CD when compared to the AGP treatment. The renewal of the intestinal epithelium is a consequence of a dynamic balance between the enterocytes production

in the crypt and the desquamation in the villus (NYACHOTI et al., 2012). Thus, the VH:CD is a useful criterion for assessing intestinal health and function (PLUSKE, 2016).

An interesting observation is that the relative spleen weight of piglets that received 30 mg of IAP when compared to those that consumed the control diet, may be associated with a greater immune system stimulation. It is evident the effect of IAP, which has the ability to attenuate the inflammatory response (CHEN et al., 2011), trigger a stimulus to the immune system in response to its presence (MUSSÁ et al., 2013) and recruit defense cells (BEUTLER & RIETSCHEL, 2003), which influenced the relative weight of this organ, as it is linked to the lymphatic system.

Overall, piglets maintained the same organ weight and were not influenced by dietary treatments, although in mammals, the ALP family consists of several isoenzymes classified into non-tissue-specific ALP (found in liver, bones and kidneys) and tissue-specific ALP (found in the bowel, placenta and germ cells) (BEUMER et al., 2003; AKIBA et al., 2007; MALO et al., 2014), which apparently demonstrates a normal state of organ development (PLUSKE et al., 2016), although it was not evaluated by immunohistopathological analysis. The toxicity generated by the challenge with ETEC has a characteristic histopathology in mammals, including liver, heart and intestinal lesions, as well as edema in the affected tissues, which may promote exacerbated organ growth (BATES et al., 2007), which did not occur in the present study.

5 CONCLUSION

The addition of 30 mg of IAP to diets promoted improvements on the growth performance and reduced the diarrhea occurrence, which can be considered a potentially effective alternative (new nutritional therapy) in replacement to AGP in diets for weaned piglets. Further studies should be conducted to better elucidate the mechanisms of action of IAP on the physiology and intestinal health of piglets. In addition, IAP influenced relative spleen weight to minimize the challenging effects of the post-weaning period and maintain an intestinal health state.

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Declaration of interest

The authors declared no conflicts of interest.

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Figure caption

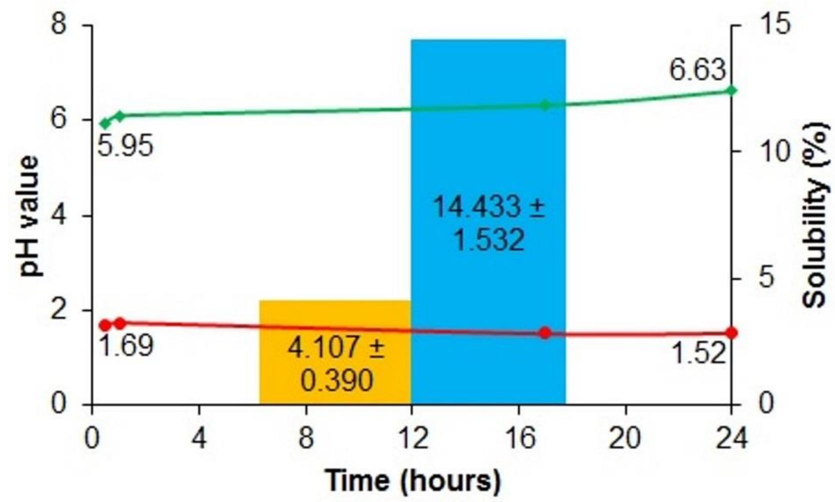

Figure 1. Solubility and effect of intestinal alkaline phosphatase *in vitro* pH modulation capacity for 24 h.

*Yellow column: IAP solubility in acidic solution. Blue column: IAP solubility in basic solution

Tables caption

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

Items	Control		Control + AGP		Control + 15 mg of IAP		Control + 30 mg of IAP	
	PI	PII	PI	PII	PI	PII	PI	PII
Grain corn 7.86%	40.10	50.75	40.07	50.72	35.99	46.64	31.88	42.53
Soybean meal 45.4%	19.75	17.84	19.75	17.84	20.48	18.57	21.22	19.31
Whey powder 12.3%	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%	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
AGP[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
Metabolizable energy (kcal/kg)	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
Analyzed 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 fiber	10.42	11.22	10.55	11.43	9.23	10.84	9.10	10.74
Acid detergent fiber	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	4,012	3,906	3,945	3,851	4,164	4,084	4,295	4,253

[1)]Experimental treatments - AGP: antimicrobial growth promoter. [2)]Experimental phases - PI e PII: pre-starter I e II. [3)]Extruded semi-whole soybean 43.16%. [4)]Nutritional levels per kg of premix, (mg/kg): Mn sulfate (120), Zn oxide (160), Fe sulfate (120), Cu sulfate (20), I (2), Sodium selenite (1.2); (mg/kg): Vitamin K_3 (12,800), Vitamin B_1 (6,400), Vitamin B_2 (16,000), Vitamin B_6 (6,400), Niacin (98,260), Pantothenic acid (32,340), Folic acid (1,920); (mcg/kg): Vitamin B_{12} (64,000), Biotin (640,000); (KIU/kg): Vitamin A (32,000), Vitamin D_3 (6,400); (IU/kg):Vitamin E (80,000). [5)]Tiamulin: 150 mg/kg of diet. [6)]Intestinal alkaline phosphatase type I-S obtained from bovine intestinal mucosa (Sigma-Aldrich Corporation).

Table 2. Additional dietary effect of intestinal alkaline phosphatase on the growth performance of piglets challenged with *Escherichia coli* K88⁺.

Items[1]	Experimental treatments[2]				SEM[3]	*P-value*[4]	1-β (%)[5]
	Control	AGP	15 IAP	30 IAP			
Pre-starter I phase (7.168 to 8.893 kg) – 25 to 35 days of age							
IBW, kg	7.171	7.168	7.168	7.167	0.050	-	-
FBW, kg	9.100	8.850	8.809	8.815	0.105	0.450	22.783
ADBWG, kg	0.192	0.168	0.164	0.164	0.009	0.450	22.252
ADFI, kg	0.249	0.239	0.220	0.210	0.010	0.282	32.886
FCR, kg:kg	1.291[b]	1.480[ab]	1.505[a]	1.287[b]	0.045	0.075	57.071
Pre-starter II phase (8.893 to 11.191 kg) – 35 to 44 days of age							
FBW, kg	11.538	11.266	10.701	11.260	0.190	0.380	25.818
ADBWG, kg	0.270[a]	0.241[ab]	0.176[b]	0.271[a]	0.011	0.004	90.208
ADFI, kg	0.379[ab]	0.383[a]	0.299[b]	0.365[ab]	0.014	0.033	70.396
FCR, kg:kg	1.410	1.374	1.615	1.390	0.062	0.359	26.793
Post-challenge phase (9.428 to 11.191 kg) – 40 to 44 days of age							
IBW, kg	9.863	9.565	8.841	9.444	0.200	-	-
FBW, kg	11.538	11.266	10.701	11.260	0.190	0.380	25.818
ADBWG, kg	0.388	0.384	0.300	0.390	0.017	0.216	37.612
ADFI, kg	0.390	0.412	0.322	0.393	0.016	0.163	42.202
FCR, kg:kg	1.005	1.066	1.121	1.012	0.037	0.615	15.947
Total period (7.168 to 11.191 kg) – 25 to 44 days of age							
FBW, kg	11.538	11.266	10.701	11.260	0.190	0.380	25.818
ADBWG, kg	0.231[a]	0.204[ab]	0.170[b]	0.218[ab]	0.008	0.040	68.176
ADFI, kg	0.314[a]	0.311[a]	0.259[b]	0.287[ab]	0.011	0.092	55.387
FCR, kg:kg	1.351	1.418	1.517	1.339	0.042	0.382	25.597

*Averages followed by different lowercase letters in row, differ according to Tukey's test at 5% and 10% probability.

[1]IBW: initial body weight; FBW: final body weight; ADBWG: average daily body weight gain; ADFI: average daily feed intake; FCR: feed conversion ratio.

[2]Experimental diets - Control: diet without feed additive; AGP: diet with the addition of antimicrobial growth promoter; IAP: diet with the addition of 15 mg intestinal alkaline phosphatase/kg of diet; 30 IAP: diet with the addition of 30 mg intestinal alkaline phosphatase/kg of diet.

[3]SEM: standard error of the mean.

[4]Significance level.

[5]Power of statistical test considering alpha = 5%.

Table 3. Additional dietary effect of intestinal alkaline phosphatase on the diarrhea occurrence in piglets challenged with *Escherichia coli* K88^{+}.

Items[1]	Experimental treatments[2]				SEM[3]	*P-value*[4]
	Control	AGP	15 IAP	30 IAP		
Pre-starter I phase (7.168 to 8.893 kg) – 25 to 35 days of age						
DO (%)	23.08	26.15	32.31	18.46	4.003	0.201
Pre-starter II phase (8.893 to 11.191 kg) – 35 to 44 days of age						
DO (%)	20.00^{ab}	28.00^{ab}	38.00^{a}	14.00^{b}	3.454	0.044
Post-challenge phase (9.428 to 11.191 kg) – 40 to 44 days of age						
DO (%)	33.33^{a}	16.66^{b}	44.44^{a}	5.56^{c}	4.754	0.009
Total period (7.168 to 11.191 kg) – 25 to 44 days of age						
DO (%)	23.31^{ab}	25.56^{ab}	36.09^{a}	15.04^{b}	2.987	0.007

*Observed proportions of diarrhea occurrence, followed by different lowercase letters in row, differ from each other by testing the difference between the lsmeans at the 5% probability level.

[1]DO (%): diarrhea occurrence.

[2]Experimental diets - Control: diet without feed additive; AGP: diet with the addition of antimicrobial growth promoter; IAP: diet with the addition of 15 mg intestinal alkaline phosphatase/kg of diet; 30 IAP: diet with the addition of 30 mg intestinal alkaline phosphatase/kg of diet.

[3]SEM: standard error of the mean.

[4]Significance level.

Table 4. Additional dietary effect of intestinal alkaline phosphatase on the blood metabolite concentrations of piglets challenged with *Escherichia coli* K88^{+}.

Items	Experimental treatments[1]				SEM[2]	*P-value*[3]
	Control	AGP	15 IAP	30 IAP		
Initial averages plasma concentrations (Baseline)						
Alkaline phosphatase, U/L	494.428	557.701	594.416	455.623	122.937	-
Urea, mg/dL	23.017	18.683	19.666	19.442	1.008	-
Glucose, mg/dL	120.578	116.803	111.980	110.705	4.757	-
Pre-starter I phase (7.168 to 8.893 kg) – 25 to 35 days of age						
Alkaline phosphatase, U/L	441.600	666.400	346.600	365.500	65.219	0.172
Urea, mg/dL	21.523	24.118	24.729	20.026	1.150	0.477
Glucose, mg/dL	118.505	128.660	114.410	122.261	3.159	0.421
Pre-challenge phase (8.893 to 9.428 kg) – 35 to 40 days of age						
Alkaline phosphatase, U/L	633.000	598.000	386.900	407.200	56.224	0.445
Urea, mg/dL	23.573	23.042	25.303	25.285	2.040	0.849
Glucose, mg/dL	97.987	97.342	88.636	93.628	4.314	0.740
Post-challenge phase (9.428 to 11.191 kg) – 40 to 44 days of age						
Alkaline phosphatase, U/L	317.600	473.100	425.900	344.100	41.881	0.609
Urea, mg/dL	18.426	19.869	22.589	21.736	1.009	0.569
Glucose, mg/dL	89.512	99.015	91.013	89.491	3.305	0.696

*Averages followed by different lowercase letters in row, differ according to Tukey's test at 5% probability.
[1]Experimental diets - Control: diet without feed additive; AGP: diet with the addition of antimicrobial growth promoter; IAP: diet with the addition of 15 mg intestinal alkaline phosphatase/kg of diet; 30 IAP: diet with the addition of 30 mg intestinal alkaline phosphatase/kg of diet.
[2]SEM: standard error of the mean.
[3]Significance level.

Table 5. Additional dietary effect of intestinal alkaline phosphatase on the intestinal morphometry of piglets challenged with *Escherichia coli* K88^{+}.

Items	Experimental treatments[1]				SEM[2]	*P-value*[3]
	Control	AGP	15 IAP	30 IAP		
Villus height (VH), µm	398.87	374.17	377.75	424.83	0.013	0.582
Crypt depth (CD), µm	162.75	194.08	171.20	158.75	0.006	0.264
VH:CD ratio	2.53	1.98	2.25	2.70	0.136	0.166

[1]Experimental diets - Control: diet without feed additive; AGP: diet with the addition of antimicrobial growth promoter; IAP: diet with the addition of 15 mg intestinal alkaline phosphatase/kg of diet; 30 IAP: diet with the addition of 30 mg intestinal alkaline phosphatase/kg of diet.
[2]SEM: standard error of the mean.
[3]Significance level.

Table 6. Additional dietary effect of intestinal alkaline phosphatase on the relative digestive and non-digestive organs weight (%) of piglets challenged with *Escherichia coli* K88^{+}.

Items[1]	Experimental treatments[2]				SEM[3]	*P-value*[4]
	Control	AGP	15 IAP	30 IAP		
Empty stomach	0.813	0.718	0.813	0.771	0.019	0.230
Empty small bowel + pancreas	4.883	5.055	5.216	4.636	0.137	0.661
Empty cecum	0.234	0.234	0.285	0.258	0.010	0.403
Empty colon	1.978	2.301	2.011	2.014	0.064	0.329
Liver + gallbladder	3.159	3.359	3.229	2.939	0.076	0.148
Spleen	0.186[b]	0.195[ab]	0.209[ab]	0.236[a]	0.006	0.043
Kidneys	0.542	0.557	0.511	0.529	0.012	0.647
Small bowel (m)	10.181	9.750	10.648	9.293	0.269	0.305

*Averages followed by different lowercase letters in row, differ according to Tukey's test at 5% probability.
[1]Small bowel (m) = measurement in meters.
[2]Experimental diets - Control: diet without feed additive; AGP: diet with the addition of antimicrobial growth promoter; IAP: diet with the addition of 15 mg intestinal alkaline phosphatase/kg of diet; 30 IAP: diet with the addition of 30 mg of intestinal alkaline phosphatase/kg of diet.
[3]SEM: standard error of the mean.
[4]Significance level.

ADDITION OF INTESTINAL ALKALINE PHOSPHATASE IN DIETS AND ITS EFFECTS ON INTESTINAL HEALTH OF PIGLETS CHALLENGED WITH ETEC K88$^+$

ABSTRACT – The goal of this study was to assess the addition of intestinal alkaline phosphatase (IAP) in diets and its effects on bacterial population count, pH of digestive tract contents, hepatic glycogen reserve (HGR), proinflammatory markers (PM) and histopathological description of weaned piglets challenged with enterotoxigenic *Escherichia coli* (ETEC) K88$^+$. The experiment involved 64 crossbred entire male piglets, weaned at 25-days-old and an average initial body weight of 7.168 ± 0.287 kg, distributed in a complete randomized block experimental design, composed of four treatments: control diet (negative control), control diet + antimicrobial growth promoter (AGP), control diet + 15 mg IAP/kg of diet and control diet + 30 mg IAP/kg of diet, eight replications with two piglets per experimental unit. All piglets were orally challenged with 6 mL of a solution containing ETEC K88$^+$ (10^6 CFU/mL). The Enterobacteriaceae counts in the cecum content were lower ($P = 0.002$) in piglets that receiving 30 mg IAP compared with those for AGP treatment. Piglets fed 30 mg IAP presented lower ($P = 0.007$) Enterobacteriaceae count in the colon when compared to the other treatments. For the Enterobacteriaceae count adhered to the mesenteric lymph nodes (MLN), there was an increase ($P = 0.006$) in piglets fed diets with AGP. Piglets fed the control diet or AGP showed greater ($P = 0.000$) lactic acid bacteria (LAB) count in the cecum content. There was a treatment effect ($P = 0.013$) on LAB count in MLN, in which piglets fed with AGP or that received 30 mg IAP had a greater count when compared to those with 15 mg IAP. The experimental treatments did not influence ($P > 0.05$) the pH of the digestive tract contents, intestinal morphology, TNF-α, COX-2 activity, TLR4 and proliferating cell nuclear antigen in the jejunum and liver, nor on HGR. Piglets that received 30 mg IAP showed a slight reduction on TNF-α in jejunum (4.17 times) and liver (1.9 times) when compared to piglets in the control group or with AGP, respectively. Based on the results, the addition of intestinal alkaline phosphatase in diets does not affect the pH of the digestive tract contents and PM of piglets, but the addition of 30 mg IAP in diets promotes a suppression of the Enterobacteriaceae population and provides an ability to mitigate intestinal injuries and maintain the homeostasis of the intestinal physiology of piglets.

Keywords: feed additives, post-weaning diarrhea, alkaline phosphatase, proinflammatory markers, antimicrobial growth promoter, bacterial populations.

1 INTRODUCTION

Effective functionality of the gastrointestinal tract (GIT) and intestinal health are important factors in determining animal performance because they involve several complex modulatory mechanisms (CELI et al., 2017). Recently, the 'health' of TGI has attracted a lot of attention, despite the lack of a clear definition of the term or its etiology, although in general terms, it covers a series of physiological and functional characteristics (PLUSKE et al., 2018). Aspects related to gastrointestinal barrier, intestinal microbiome, immune system, psychiatric disorders, management practices and nutritional management are critical factors in survival and intestinal health of piglets (KELLY et al., 2015; HONDA & LITTMAN, 2016; JAYARAMAN & NYACHOTI, 2017; LIAO & NYACHOTI, 2017; LIU et al., 2018), as they are continually challenged by factors responsible for histological and physiological changes in the small intestine and/or are challenging factors to piglets (PLUSKE et al., 2018).

As auxiliary and complementary mechanisms to mitigate these effects, piglets have enteroendocrine cells that play important roles, such as pathogen detection, synthesis and release of neuropeptides (MOESER et al., 2017), recognition of pathogenic signaling molecules and interleukin secretion and growth factors (SCHIERING et al., 2014; MOESER et al., 2017; PLUSKE et al., 2018), which have a diverse range of physiological functions and important immunomodulatory properties. Furthermore, intestinal microbiota plays a role in the synthesis of beneficial nutrients, action on the deleterious effects of inflammation and subclinical/clinical pathologies (CELI et al., 2017), in addition to influencing the functional diversity of IgA-producing cells that carry the CD4 antigen (HONDA & LITTMAN, 2016).

However, pathogenic infection is one of the main challenges that affect the intestinal health of piglets (SUN & KIM, 2017). Any discussion in this critical window of transition should include the potential impacts of enterotoxigenic *Escherichia coli* (ETEC) strains (YI et al., 2016; GRESSE et al., 2017), including mainly F4 $(K88)^+$ (FAIRBROTHER et al., 2017; PAN et al. al., 2017) and $F18^+$ (FAIRBROTHER et al., 2017; SUN & KIM, 2017). ETEC is capable of synthesizing a wide range of enterotoxins and thermostable peptide toxins (ABUBAKAR et al., 2017). *E. coli* post-weaning diarrhea, also called post-weaning enteric colibacillosis is a crucial factor that can cause piglet mortality (GRESSE et al., 2017; SUN & KIM, 2017). As a consequence, the basic mechanism of enterotoxins is to impair the epithelial cell function of the small intestine and increased dehydration (SUN & KIM, 2017).

Of course, and in context, the attention of the swine industry, the understanding and appreciation of factors and measures related to intestinal health has increased considerably in

recent years, mainly due to changes that reduce the use of antimicrobials, especially performance-enhancing antibiotics, with the potential to kill or prevent the growth of pathogenic and beneficial microorganisms (GRESSE et al., 2017). In this sense, a wide range of products such as feed additives, nutritional strategies and available management practices that aim to promote or improve GIT health have been investigated (ADEWOLE et al., 2016; JAYARAMAN & NYACHOTI, 2017).

Studies have reported the use of IAP and its effects on intestinal health, through decreased inflammation in the colon (ALAM et al., 2014), reduction of inflammatory activity of TNF-α levels (MOSS et al., 2013), normalization of neutrophilic marker expression (MARTÍNEZ-MOYA et al., 2012), ability to promote bacterial growth and normalize the intestinal microbiome (MALO et al., 2014), modulation of intestinal pH (BRUN et al., 2014) and ability to dephosphorylate the nucleotide uridine diphosphate (MOSS et al., 2013).

Our hypothesis was that the addition of IAP in diets would promote intestinal health by reducing inflammatory processes in the gut and consequently provide a benefit on the immunity and intestinal damage of piglets in the nursery phases. Thus, the aim of this study was to evaluate the addition of intestinal alkaline phosphatase in diets and its effects on intestinal health by counting bacterial populations in the intestinal contents and adhered to mesenteric lymph nodes, pH of digestive organ contents, hepatic glycogen reserve, immunohistochemistry of proinflammatory markers in the liver and intestinal epithelium and histopathological description of the intestinal epithelium of weaned piglets challenged with *E. coli* $K88^+$.

2 MATERIAL AND METHODS

The experiment was carried out in the Swine Sector of the Experimental Farm Professor Antonio Carlos dos Santos Pessoa of the State University of Western Paraná - UNIOESTE, Campus of Marechal Cândido Rondon/Paraná, Brazil. The piglets were carefully managed to avoid unnecessary discomfort and all experimental procedures were approved by the UNIOESTE Research Ethics Committee (No. 13/19 - CEUA).

All procedures of euthanasia for the animals were performed by electronarcosis, in compliance with the Normative Resolution No. 37 of February 15, 2018 of CONCEA-Brazil, which establishes the Guidelines of the Practice of Euthanasia of the National Council for Control of Animal Experimentation.

2.1 Experimental design, animals, housing and diets

A total of 64 crossbred piglets (Landrace x Large White), entire male weaned at 25-days-old with an average initial body weight of 7.168 ± 0.287 kg were distributed in a randomized complete block design consisting of four treatments repeated twice in the time, totaling 32 experimental units (EU), with two animals per EU.

At the beginning of the experimental period, the animals were weighed and identified with numbered ear tags and housed in a masonry nursery shed and ceramic roof tiles, consisting of suspended pens (1.545 m²), with polyethylene plastic flooring, equipped with nipple-type drinking fountains and gutter-type feeders, arranged in two rows, divided by a central corridor, where they remained for a period of 19 days.

The ambient temperature and relative humidity were recorded using a data logger with digital display (Vketech brand, temperature instruments model), which was installed in the center of the experimental building. The minimum recorded temperature of the internal environment was 19.1 ± 5.2ºC and the maximum was 29.7 ± 5.5ºC. The nursery shed 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 growth phases I and II, following the nutritional recommendations proposed by Rostagno et al. (2017). The experimental treatments (Table 1) were composed of a control diet (negative control), control diet + antimicrobial growth promoter (AGP, 150 mg tiamulin/kg of diet), control diet + 15 mg IAP/kg of diet and control diet + 30 mg IAP/kg of diet.

2.2. Sample collection, preparation and analytical procedure

2.2.1. Process of IAP microencapsulation

The methodology used for 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. After the dilution step, the temperature of the mixer was reduced because there was information on the possibility of inactivating the enzyme with the use of high temperatures. The first PGE emulsifier was added with the mixer at a temperature of approximately 5°C, in which it was chosen because it showed a greater tendency to solidify in contact with the product, in this case, when added the first agglomerates were formed. After further cooling the PA was added, which gave greater structure and finished the agglomeration process and protection of the enzyme. Because it is a process of low production, an industrial stirrer was used and the PGE was added at 55°C in the first step and the PA at 65°C taking into consideration the melting points of each component. As seen on day, the enzyme in contact with the water remained intact showing that there was no RS available for this contact. The final composition of the vehicle used in the microencapsulation process presented the following proportions: 50% RS, 30% PGE + 20% PA.

2.2.2. Process of evaluation of post-microencapsulation IAP activity

IAP was microencapsulated in 50% RS + 50% propylene glycol ester (IAP I) or microencapsulated in 50% RS + 50% propylene glycol ester with palmitic acid (IAP II). After the microencapsulation process, the IAP activity on adhesion and the phagocytic activity of the equine bronchoalveolar macrophage was evaluated. The microencapsulated enzymes were properly weighed and diluted in 50% ethanol for 24 h, kept under stirring in an oven at 37°C.

The procedures tested were IAP in the forms: pure lyophilized (IAP), IAP I and IAP II induced or not with *E. coli* LPS. As control procedures were used the alveolar macrophage (AM), AM + phosphate-buffered saline (PBS) and AM + IAP groups, without the addition of LPS (microcytic activity inducer). Macrophages were isolated from bronchoalveolar lavage (BAL) from a healthy mare with 360 kg of body weight. The animal was fasted for 6 h prior to collection to perform sedation using intravenous (IV) detomidine (Eqdomin, 0.02 mg/kg, Ourofino, São Paulo, Brazil) and pethidine chloride IV (Dolosal, Cristália, São Paulo Brazil). BAL fluid collection was performed with a flexible silicone catheter of 300 cm length and 8

mm diameter (V-PBAL-300, Cook Vet Products, Hamburg, Germany). It was introduced via the nasotracheal tube until it was lodged in a bronchus, when the cuff at the distal end was inflated with 10 mL of air. An amount of 500 mL of sterile saline solution (0.9% NaCl) preheated to 37°C was used (HOFFMAN, 2008), infused into 125 mL aliquots and aspirated after each aliquot. The collected material was stored on ice in tubes until it reached at the laboratory.

In the laboratory, BAL fluid was centrifuged at 340 g for six minutes at 4°C (MICHELOTTO et al, 2010) and the pellet obtained was suspended in the animal's own supernatant. Total nucleated cell count was performed in the Neubauer chamber, in two opposite quadrants, and cell viability was verified through trypan blue. From the cell pellet, 10 µL was used for slides mounting, which were stained with the Romanowski technique (Panotic Rapid, Laborclin, Paraná, Brazil) for differential cell count in 1000x magnification. Two 2 mL aliquots of BAL fluid supernatant were separated in the centrifugation phase and frozen at -20°C for further analysis.

After counting the total number of cells obtained in the BAL fluid, the cell suspension was adjusted to the concentration of 5 x 10^6 viable cells/mL. An amount of 100 µL of the adjusted suspension was plated into 96-well microplate wells (in triplicate) to analyze adhesion and phagocytic activity (one plate for each assay). The plates were incubated for 1 h at 37°C. After this period, the supernatant was discarded, leaving only the AM adhered to the plastic. After the isolation of the AM, the phosphate-saline buffer (PBS, control group) and ETEC lipopolysaccharide (LPS, 100 ng/mL) inflammatory inducers were added in the amount of 50 µL. The plates were incubated for an additional 60 minutes in an oven at 37°C, then the treatments were carried out: T1: PBS; T2: AM + PBS; T3: AM + IAP; T4: AM + LPS; T5: AM + LPS + IAP; T6: AM + IAP I; T7: AM + LPS + IAP I; T8: AM + IAP II and T9: AM + LPS + IAP II.

After 1 h of incubation of the treatments, the supernatant containing the treatments on the plate was discarded, the wells were washed twice with PBS and the adherent cells were then fixed with 50% methanol for 10 minutes at ambient temperature. After this time, the supernatant was discarded and 100 µL of 0.2% Giemsa dye (Sigma-Aldrich, St. Louis, Missouri, United States) was added to each well for 40 minutes at ambient temperature. The wells were again washed twice with PBS and 200 µL of 50% methanol was added. After 30 minutes, the resulting solution had its absorbance read in a spectrophotometer with a wavelength corresponding to 550 nm (Sunrise, Tecan, Switzerland). This test was adapted from the technique of Rosen &

Gordon (1987). The final adhesion result was corrected by the percentage of alveolar macrophages in the BAL fluid (absorbance x % macrophages).

To analyze the phagocytic activity of the alveolar macrophage, after the macrophage plates had their wells washed twice with PBS, as in the previously mentioned assay, 100 µL PBS and 20 µL zymosan (Sigma-Aldrich, St. Louis, Missouri, United States) were added to each well, stained with neutral red (6.7 mg/mL, Sigma-Aldrich, St. Louis, Missouri, United States) and incubated at 37°C for 30 minutes. Then, the supernatant was discarded and the cells were fixed with Baker's solution (4% formaldehyde, 2% sodium chloride, 1% calcium acetate, aqueous solution) for 30 minutes at 37°C. The supernatant was discarded and 100 µL PBS was added to perform centrifugation at 400 g for 5 minutes. Then, after discarding the supernatant, the neutral red dye was solubilized with 200 µL extraction solution (10% glacial acetic acid, 40% ethanol in aqueous solution) and incubated for 30 minutes. After this period, the absorbance was measured at 550 nm. This assay was adapted from the method described by Dyrynda et al. (1998). The final result of phagocytic activity was corrected by the percentage of alveolar macrophages in the BAL fluid (absorbance x % macrophages).

It was observed that macrophage adhesion was higher for treatments composed of AM + LPS + IAP I and AM + LPS + IAP II. However, the phagocytic activity related to macrophage adhesion was lower for the groups AM + LPS + IAP, AM + LPS + IAP I and AM + LPS + IAP II compared to the other treatments. The group with AM + LPS + IAP II presented the lowest phagocytic activity. In short, pure or microencapsulated IAP showed activity even after handling and microencapsulation process, respectively. After the pilot analysis of the enzymatic activity, the IAP II microencapsulation method was selected to continue processing the enzyme and its addition to the diet.

2.2.3 Bacterial strain and challenge procedure

Four days before slaughter, the 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 of ETEC K88$^+$ provided by the Laboratory of the Mercolab (Cascavel, PR, Brazil). ETEC was multiplied in brain heart infusion broth for 18-24 h at 36 ± 1°C until reaching the concentration of 1.0 x 10^9 CFU/mL. Subsequently, serial dilution was performed in saline solution (0.9% NaCl) up to the concentration of 1.0 x 10^6 CFU/mL. The infection via in piglets was at the back of the oral cavity using a syringe. The rich solution in ETEC was

slowly dripped into the piglet's throat so that the swallowing reflex was triggered and the inoculant's passage into the lungs was minimized (OWUSU-ASIEDU et al., 2003).

2.2.4 Samples of intestinal epithelium and liver

At 19 d of experimentation, six animals from each experimental treatment were slaughtered (after a six-hour fasting) following humane slaughter methods (electronarcosis followed by exsanguination) to collect data and biological samples for histopathological analysis of the intestinal epithelium, lymph nodes and liver, measuring the pH of the digestive tract contents and counting of bacterial populations in the contents of jejunum, cecum, colon and adhered lymph nodes. For the expression analysis of factors related to inflammation, segments of the jejunum and liver were also collected for immunohistochemical evaluation. The choice of animal to be slaughtered was according to body weight (BW), being used the one that presented the closest BW to the average of the treatment. Fasting was performed to reduce the presence of residues in the organs, to facilitate their handling and to avoid damage to the tissues that were used for histopathological and immunohistochemical analyses.

2.2.5 pH of digestive tract contents

Measurement of the pH of the stomach, jejunum, cecum and colon contents was performed with the aid of a digital pH meter (Hanna Instruments Inc., Rhodes Island, USA, model HI 99163) through the insertion of a unipolar electrode, adopting the methods described by Manzanilla et al. (2004). Ligatures were performed at the ends of the mentioned organs, so that there was no mixture of contents and impairment of pH results. Access to the contents present in the stomach was made from an incision in the esophageal region (approximately two centimeters from the esophagus) and the pH was measured after homogenization of the digestive contents. After digesta homogenization 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 cecum and colon (GUO et al., 2001).

2.2.6 Microbiological analyses

After slaughter, samples of the contents of the jejunum, cecum and colon were collected, as well as the mesenteric lymph nodes (MLN), which were destined for population counts of

Enterobacteriaceae and lactic acid bacteria. The samples were packed in sterile plastic containers, identified and then stored and transported under refrigeration for laboratory analysis. The MLN were aseptically macerated with the aid of a mortar and pestle, previously autoclaved. Subsequently, one gram of sample of the contents of the digestive tract and macerated MLN was transferred to identified sterile tubes and submitted to serial dilution in saline solution (0.9% NaCl). The dilution 10^{-1} (1 g of sample with 9 mL of saline solution) was homogenized in vortex for 30 seconds. The remaining dilutions, up to 10^{-6} (for cecum and colon) and 10^{-5} (for MLN), were homogenized in vortex for 10 seconds. An aliquot of 100 µL of each dilution was seeded by surface spreading in culture medium for Enterobacteriaceae (EMB levine agar, Kasvi) and lactic acid bacteria (MRS agar, Acumedia) in sterile plastic petri dish suitable for its growth according to the methodology described by Weedman et al. (2011). To detect Enterobacteriaceae populations, the inoculum plates were incubated in aerobic greenhouses overnight at 37°C for 24 h. To detect lactic acid bacterial populations, inoculum plates were incubated in anaerobic greenhouses overnight at 37°C for 44 h.

2.2.7 Histopathology of the intestinal epithelium and proinflammatory markers on liver and intestinal epithelium

To assess the structures of the intestinal epithelium, immediately after the removal of the organs, 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 Histopathology Laboratory of the Mercolab (Cascavel, PR, Brazil) where they were paraffin-embedded and microtomized for slides mounting. The paraffin blocks containing the samples were cut in a microtome (ANCAP 78), sections were performed and transferred to the slides. The slides were stained with hematoxylin and eosin for histopathological description (GAO et al., 2000) and PAS (Periodic acid-Schiff) staining for hepatic glycogen reserve (TUIN et al., 2006). The analyses described above were evaluated with the aid of an optical microscope and by computer program ZEN 2.0 Image software.

For proinflammatory reactivity by immunohistochemical evaluation (TNF-α, COX-2, TLR4 and PCNA), the same samples used for structural analysis were used to create the blocks

by the tissue microarray (TMA) technique, described by Engracia Filho et al. (2017). Paraffin blocks containing 24 sample fragments were made to subsequently prepare the slides. COX-2 immune expression was assessed using the polyclonal anti-Cox-2 antibody (Dako, Glostrup, Denmark). Subsequently, the slides were scanned on the Axio Scan.Z1 scanner (Carl Zeiss®, Jena, Germany) and analyzed using Image Pro Plus 4 software (Media Cybernetics Inc., Rockville, USA). The percentage area immunolabeled with COX-2 (μm^2) was calculated by evaluating seven images for each replicate, according to the methodology of Alvares et al. (2018). 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. The TNF-α-positive cells were counted using images obtained from the Olympus BX40 microscope with a 40x objective lens. Five random fields were photographed of the jejunum and liver in each replicate, and subsequently the average count of immunolabeled cells was obtained. Immunohistochemical analysis of TLR4 (%) were made using a TLR4/CD284 polyclonal antibody (Product PA-23125, ThermoFisher Scientific) at 1:100 dilution. Immunohistochemical analysis of PCNA (%) using anti-PCNA polyclonal antibody (Product PA5-32541) at dilution 1:100. The parameters analyzed in the morphology of the intestinal epithelium were the presence of infiltrate, hyperemia, desquamation, coccidiosis, lumps, rods, cysts, mucus, goblet cells and necrosis.

2.2.8 Calculations and statistical analyses

Before evaluating the result of analysis of variance (ANOVA), the standardized residuals analysis of Student (RStudent) was performed in order to identify influential observations or outliers. The criterion adopted for identification of outliers was based on a normal distribution curve; RStudent values greater than or equal to three standard deviations were considered as influential. The normality of experimental errors and the homogeneity of error variances between treatments for the several variables were previously evaluated using the Shapiro-Wilk and Bartlett tests, respectively.

For the characteristics analyzed, the statistical model used was: $Y_{ijk} = m + T_i + b_j + \varepsilon_{ijk}$. The effects of the factors included in the model were described by: 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; m = 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 level i, block j and replication k. For the counting characteristic of bacterial populations,

the data were transformed into logarithm (base 10).

For statistical analysis of histopathological description of infiltrate, hyperemia, epithelial desquamation and tissue necrosis, the Generalized Linear Model (GLM) was adjusted with the Gaussian Inverse distribution and log binding function. For bacterial lump and mucus characteristics, the GLM was adjusted with the negative binomial distribution and log binding function. For the goblet cell variable, the GLM was adjusted with the Normal distribution and log binding function. GLM 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 together with the graphical analysis of residue adherence. Average comparisons were performed using a test of the difference between the lsmeans, through the χ^2 statistic.

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. All statistical analyses were performed using the procedures of the statistical software “SAS University Edition” (SAS Inst. Inc., Cary, NC, USA). Data were presented as means with standard error of the mean.

3 RESULTS

3.1 Counts of intestinal and adhered microbial populations in mesenteric lymph node, and pH of digestive tract contents

To assess whether IAP affects intestinal colonization, the number of Enterobacteriaceae and lactic acid bacteria (LAB) adhered to the intestinal mucosa and mesenteric lymph nodes (MLN) was investigated (Table 2). Enterobacteriaceae counts on cecum content were lower ($P = 0.002$) for treatment piglets that received 30 mg IAP compared to those on AGP and 15 mg IAP treatments. Piglets fed control diets, AGP and 15 mg IAP had greater ($P = 0.007$) Enterobacteriaceae counts in the colon when compared to those with 30 mg IAP. For Enterobacteriaceae count adhered to the MLN, there was an increase ($P = 0.006$) in piglets fed diets with AGP. Piglets fed the control diet or AGP showed higher ($P = 0.000$) LAB count in the cecum content. There was a treatment effect ($P = 0.013$) on LAB count in MLN, where AGP-fed piglets and that received 30 mg IAP had a greater count compared to those with 15 mg IAP.

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

3.2 Histopathological description of the piglet jejunum

There was no effect ($P > 0.05$) of the experimental treatments on the histopathological description of the challenged piglet jejunum with ETEC $K88^+$ (Table 4).

3.3 Proinflammatory markers on liver and intestinal epithelium, and hepatic glycogen reserve

To verify the effect of IAP on the ability to attenuate inflammatory processes in the jejunum and liver, and to determine the ability to store energy reserves in the liver, the tumor necrosis factor alpha (TNF-α), cyclooxygenase 2 (COX-2) enzyme activity, Toll-like receptor 4 (TLR4), proliferating cell nuclear antigen (PCNA) and the hepatic glycogen reserve (HGR) of piglets were evaluated. However, there was no effect of the treatments on the TNF-α 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 ($P = 0.243$), proliferating cell nuclear antigen (PCNA) in the jejunum ($P = 0.668$) and liver ($P = 0.127$), nor on HGR (P

= 0.236). Piglets that received 30 mg IAP showed a slight reduction on TNF-α in jejunum (4.17 times) and liver (1.9 times) when compared to piglets in the control group or with AGP, respectively.

4 DISCUSSION

4.1 Counts of intestinal and adhered microbial populations in mesenteric lymph node, and pH of digestive tract contents

It was also evident that average microbial population count values for Enterobacteriaceae in cecum and MLN were prevented in piglets fed diets absent feed additive, which may be related to the fact that piglets developed a tolerance to bacterial pathogens (CHEN et al., 2011). It is possible that piglets that received AGP had the intestinal barrier impaired by ETEC infection (DAUDELIN et al., 2011), which increased the permeability to toxins such as endotoxin or LPS of Gram-negative bacterial cell walls (LAMBERT, 2009), as IAP added in 30 mg in the diet had the ability to normalize the intestinal microbiome for the growth of Enterobacteriaceae (ALAM et al., 2014).

Based on the body's defense 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). Therefore, understanding the variant effects of AGP on the gut-associated tissue microbiome is of vital importance to maintain metabolic homeostasis and intestinal health.

Probably, piglets that consumed diets containing AGP suffered a reperfusion of blood into the intestinal tissue, which caused a drastic fluctuation of oxygen levels (not determined in the present study) exacerbating cell stress, cell apoptosis and, finally, leading to a breakdown of the epithelial barrier marked by increased intestinal permeability and bacterial translocation to the MLN (EARLEY et al., 2015). The axis of the intestinal lymph nodes plays a key role in the symbiotic relationship between the intestinal microbiota and the host immune system (KAMADA & NÚÑEZ, 2013), which surprised us with the counts obtained for the control group piglets, supposedly due to the piglet's ability to present or not receptors for the different types of adhesins (SUM & KIM, 2017).

Regarding to the average count values for LAB in the cecum, it was clear about the reduction of IAP activity, being verified only non-specific alkaline phosphatase isoforms in the portion of the cecum (LALLÈS, 2014). Supposedly, IAP activity 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. AGP alter 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). Recent findings suggest that AGP in diets reduce the abundance of some Gram-positive genders, but do not induce changes in the phylum level in pigs (KIM et al., 2012).

Data have also shown that weaning causes fluctuation in the predominant bacterial populations in the GIT (WEEDMAN et al., 2011), as the number of predominant species has increased slightly from cecum to colon, suggesting a more complex microbiota and that digestive flow in the large intestine promotes greater bacterial diversity. In general, IAP added in the amount of 30 mg modulates the microbiota ecosystem, suppressing the population of Enterobacteriaceae.

The results of the present study suggest a lower role for the pH of the lumen in the modulation of fecal microbiota (ZHANG et al., 2010). This observation is inconsistent as a higher pH (7.2-7.8) is speculated to provide an ideal environment for ETEC colonization on the surface of the villi, resulting in early diarrhea in piglets (OWUSU-ASIEDU et al., 2003; GONZALES et al., 2013) or when associated by the IAP's ability to modulate the intestinal pH (AKIBA et al., 2007; BRUN et al., 2014; MALO et al., 2014). However, the growth of ETEC can be verified in a wide range of pH (4.5-9.0) (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 (SPRING et al., 2000; MISSOTEN et al., 2015; SUIRYANRAYNA & RAMANA, 2016); however, due to the results obtained it was not possible to corroborate this hypothesis.

4.2 Histopathological description of the piglet jejunum

The effect on intestinal morphology cannot be analyzed 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. The results indicated (although not significantly) a mitigation of tissue injury in piglets fed 30 mg IAP or AGP.

Apparently, piglets that received IAP in diets showed a tendency to reduce tissue necrosis and this can be explained by its action in reducing inflammatory events to support the immune system (LALLÈS, 2014). In addition, the data suggested that IAP attenuated cell loss due to epithelial desquamation related to the control group, as well as piglets that received AGP, which may be an indication of reduction of inflammatory alteration. For piglets fed AGP or 30 mg IAP, the findings are consistent when it was related to hyperemia with epithelial desquamation and tissue necrosis because the most common occurrence of hyperemia is observed in acute/pathological inflammation.

When the histopathological description for inflammatory infiltrate was analyzed, an occurrence of cell cluster around the epithelium was found to replace functional tissue in piglets of the negative control group. It was also verified the mucus discrimination in piglets that consumed the control diet or 15 mg IAP and absence in piglets that received AGP or 30 mg of IAP, which can be explained by bacterial challenge (intestinal infection), usually accompanied by diarrhea and/or damage to the jejunal mucosa (LÓPEZ-COLOM et al., 2019). However, prolonged use of antibiotics can cause gastroenteritis problems (KIM et al., 2019) and concomitantly promote an increase in the presence of intestinal mucus, a fact not observed in the present study. Thus, goblet cells increase the production of mucus in situations of intestinal damage and alteration of the intestinal microbiota (SICARD et al., 2017; OKUMURA & TAKEDA, 2017), since these occurrences can cause a reduction in the mucus layer and promote the action of pathogenic bacteria that cause mucosal rupture, which consequently promotes an increase in goblet cells.

However, previous studies have reported that the challenge with ETEC in piglets was not associated with significant macroscopic lesions or morphological changes in the intestinal mucosa (NAGY & FEKETE, 1999; DUBREUIL et al., 2016), but revealed lesions of dehydration, stomach and small intestine dilatation, gastric infarcts in the stomach mucosa and congestion of the small intestine and colon (MADEC et al., 2000), in addition to shock syndrome with hemorrhagic gastroenteritis, congestion, renal hemorrhage, and thrombi in the stomach and small intestine (FAUBERT & DROLET, 1992; MOXLEY et al., 1998).

4.3 Proinflammatory markers on liver and intestinal epithelium, and hepatic glycogen reserve

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 tumor necrosis factor α (ANDRADA et al.,

2014; KUNANUSORNCHAI et al., 2016; WALTER et al., 2019). However, in previous studies it was evidenced that this enzyme was able to act in the repair of intestinal mucosal lesions (HAWORTH et al., 2005) and play an important role in pathophysiological processes (CHO & CHAE, 2004).

The fact that piglets fed with 30 mg IAP show an average COX-2 value in the jejunum of 2.2 times higher than those receiving 15 mg IAP can be attributed to the mitigation of intestinal injuries proven by histopathological description, since the inhibition of COX-2 can lead to damage in the small intestine (HAWORTH et al., 2005), reduce the maintenance of the stomach integrity (WALLACE et al., 2000) and the small intestine (SIGTHORSSON et al., 2002). However, piglets fed 15 mg IAP showed a reduced effect (numerically evidenced) in the COX-2 activity, it is a mechanism that we do not know due to the results of intestinal morphology.

In a study conducted by Kim et al. (2016), who tested the effect of acetylsalicylic acid supplementation in piglet diets and also found no differences between treatments for COX-2 content in the liver. In contrast to our findings, Kunanusornchai et al. (2016) obtained suppression of COX-2 expression in rabbit synoviocytes when orally fed chitosan oligosaccharide. To our knowledge, there seem to be no reports in the literature about studies that determined the COX-2 concentration in piglets fed with IAP.

According to Lauridsen et al. (2010), at least some variation in the COX-2 expression in intestinal samples from 28-day-old piglets can be attributed to the use of antibiotics, which is not in accordance with the results of this study since all treatments maintained the same variation. However, the COX-2 enzyme may present greater expression in the distal parts of the intestine, differing from COX-1 which is relatively uniform along the GIT (LAURIDSEN et al., 2010).

TNF-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 with IAP, although a slight reduction was observed in piglets that received 30 mg IAP, which is in agreement with Beumer et al. (2003), who reported a difference in TNF-α release among piglets that were treated with LPS or LPS + IAP. In our study, the challenge with ETEC influenced an evident numerical increase in the production of proinflammatory cytokine TNF-α in the jejunum of piglets that received the control diet or AGP, suggesting an impaired effect on intestinal health (LEE et al., 2012) because TNF-α is involved in the acute phase response promoting the increase of IL-6 (LÓPEZ-COLOM et al., 2019) and highly expressed in the

chronically inflamed gut (BISCHOFF et al., 2014), and this was also related to the results of tissue necrosis.

However, TNF-α is involved in cell removal processes of intestinal epithelium (BISCHOFF et al., 2014), which would be more evident in epithelial desquamation, but for a more complete verification of intestinal damage or exacerbated increase in TNF-α it is 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). Wan et al. (2019), who tested the effect of low-molecular-weight chitosan in piglet diets challenged with ETEC and found a reduction for TNF-α in the small intestine of piglets fed with the feed additive, which is also in agreement with Ren et al. (2019).

The fact that piglets fed 30 mg IAP show reduced results of TNF-α in the liver and intestine suggests the role of IAP in reducing the induction of inflammatory responses (BEUMER et al., 2003), which in turn attenuates the production of plasma acute phase proteins by the liver (BAUMANN & GAULDIE, 1994) and cell apoptosis because the TNF-α family can activate the apoptotic cascade leading to cell death via caspase-8 recruitment (WAN et al., 2019). These results indicated that the addition of 30 mg IAP in the diet of piglets challenged with ETEC can partially improve intestinal integrity and liver damage, suppressing the production of TNF-α induced by bacterial challenge, corroborating the findings of Pu et al. (2018) and Peng et al. (2019), who tested different feed additives in diets for weaned piglets.

The liver plays an important role in regulating glucose production and glycogen synthesis (FAIRBEG 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, the reason why control group piglets showed 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).

It is possible that the bacterial challenge affected or induced the energy metabolism of piglets that received the control diet because there was an increase in the depletion route of glycogen content, which is a factor that activates several important mechanisms to maintain homeostasis normal blood glucose level (ROACH et al., 2012), emphasizing the importance of a fast energy supply for several metabolic purposes (THEIL et al., 2014). In general terms, piglets that are challenged or show acute clinical signs of disease can result in a decrease in the glucose concentration (WILSON et al., 1972) and with that there is a reduction in HGR, which

has also been verified in intracellular energy levels in the form of adenosine triphosphate of piglets when subjected to the post-weaning challenge (NOVAIS et al., 2019).

Toll-like receptors (TLRs) 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 K88$^+$ are still unexplored. However, piglets fed 30 mg of IAP showed a slight suppression in TLR4 activation when compared to those receiving AGP. This indicates that IAP when added to 30 mg in the diet of piglets challenged with ETEC K88$^+$ can attenuate the increase in TLR4 concentration, since inflammatory processes of pathogenic origin involve increased TLR signaling (DUBREUIL, 2017).

In the present study, the *in vivo* challenge model based on ETEC K88$^+$ caused an immune stimulation through the TLR4 signaling pathway (LUISE et al., 2019), but some mechanisms in the expression and function of TLRs in weaned piglets challenged with ETEC K88$^+$ still need further investigations and elucidation (XU et al., 2014). Our findings are in accordance with the results reported by Zhang et al. (2015), who tested the use of a probiotic (*Lactobacillus rhamnosus* ATCC 7469) and showed a reduction of intestinal TLR4 in piglets that received the probiotic-based treatment, which also corroborates the results of Qin et al. (2018), who evaluated the effect of glutamate in piglets challenged with lipopolysaccharide.

Immune changes in piglets during TLR4 signaling under stress by ETEC K88$^+$ in liver damage are poorly known. TLR4 expression has tissue specificity and is related to ETEC resistance in weaned piglets (WANG et al., 2013). However, proliferative and apoptotic changes during the TLR4 signaling mechanism need further description at the hepatic level (HUANG et al., 2017). Therefore, there is no plausible response to the notable increase in TLR4 concentration in the liver of piglets that received 30 mg of IAP, although TLR4 and IAP expression increase in the presence of LPS (ABASHT et al., 2008; CHEN et al., 2010). However, IAP is a local defense factor of intestinal mucosa (LALLÈS, 2014) and only specific isoforms with unknown function are found in the liver (FAWLEY & GOURLAY, 2016; HAARHAUS et al., 2017).

Taken together, even though increased TLR4 signaling may play a key role in liver injury (HUANG et al., 2017), there is not enough evidence for this statement in the present study because piglets fed 30 mg of IAP 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). Research on TLR4 modulation in piglets when fed with IAP and subjected to a bacterial challenge is lacking so far, since Leng et al. (2014) observed different regulation peaks for pro-

inflammatory mediators and genes related to TLR4 signaling in piglets fed aspartate-based diets and subjected to challenge with LPS.

To our knowledge, there are no reports that analyzed the proliferative cell nuclear antigen (PCNA) in studies involving piglets fed IAP and challenged with ETEC $K88^{+}$. 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).

In a study conducted by Verdile et al. (2019), who evaluated the evolution of intestinal stem cells in pigs from birth to weaning and found proliferative cells, although at low frequency, along the villi and on the luminal surface of the colon. 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 to the control group. However, in this study, the challenge in which the piglets were subjected was poorly able to promote cell regeneration, which implies further investigations to elucidate the mechanisms of action of ETEC $K88^{+}$ on activation of the PCNA markers.

5 CONCLUSION

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 IAP in diets promotes a suppression of the Enterobacteriaceae population and provides an ability to mitigate intestinal injuries and maintain the homeostasis of the intestinal physiology of piglets.

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Declaration of interest

The authors declared no conflicts of interest.

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

Items	Control		Control + AGP		Control + 15 mg of IAP		Control + 30 mg of IAP	
	PI	PII	PI	PII	PI	PII	PI	PII
Grain corn 7.86%	40.10	50.75	40.07	50.72	35.99	46.64	31.88	42.53
Soybean meal 45.4%	19.75	17.84	19.75	17.84	20.48	18.57	21.22	19.31
Whey powder 12.3%	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%	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
AGP[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
Metabolizable energy (kcal/kg)	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
Analyzed 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 fiber	10.42	11.22	10.55	11.43	9.23	10.84	9.10	10.74
Acid detergent fiber	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	4,012	3,906	3,945	3,851	4,164	4,084	4,295	4,253

[1)]Experimental treatments - AGP: antimicrobial growth promoter. [2)]Experimental phases - PI e PII: pre-starter I e II. [3)]Extruded semi-whole soybean 43.16%. [4)]Nutritional levels per kg of premix, (mg/kg): Mn sulfate (120), Zn oxide (160), Fe sulfate (120), Cu sulfate (20), I (2), Sodium selenite (1.2); (mg/kg): Vitamin K_3 (12,800), Vitamin B_1 (6,400), Vitamin B_2 (16,000), Vitamin B_6 (6,400), Niacin (98,260), Pantothenic acid (32,340), Folic acid (1,920); (mcg/kg): Vitamin B_{12} (64,000), Biotin (640,000); (KIU/kg): Vitamin A (32,000), Vitamin D_3 (6,400); (IU/kg):Vitamin E (80,000). [5)]Tiamulin: 150 mg/kg of diet. [6)]Intestinal alkaline phosphatase type I-S obtained from bovine intestinal mucosa (Sigma-Aldrich Corporation).

Table 2. Additional dietary effect of intestinal alkaline phosphatase on the Enterobacteriaceae and lactic acid bacteria counts (Log_{10} CFU/g) of piglets challenged with *Escherichia coli* K88^{+}.

Items	Experimental treatments[1]				SEM[2]	*P-value*[3]
	Control	AGP	15 IAP	30 IAP		
Counts of Enterobacteriaceae (Log_{10} CFU/g)						
Cecum	7.34^{bc}	8.06^{a}	7.77^{ab}	7.02^{c}	0.130	0.002
Colon	7.22^{a}	7.56^{a}	7.04^{a}	6.18^{b}	0.140	0.007
Mesenteric lymph nodes	4.21^{b}	5.04^{a}	4.47^{b}	4.50^{b}	0.116	0.006
Counts of lactic acid bacteria (Log_{10} CFU/g)						
Cecum	9.22^{a}	9.17^{a}	8.73^{b}	8.33^{c}	0.060	0.000
Colon	8.45	8.81	8.82	8.76	0.130	0.240
Mesenteric lymph nodes	6.15^{ab}	6.24^{a}	5.64^{b}	6.30^{a}	0.116	0.013

*Averages followed by different lowercase letters in row, differ according to Tukey's test at 5% probability.
[1]Experimental diets - Control: diet without feed additive; AGP: diet with the addition of antimicrobial growth promoter; IAP: diet with the addition of 15 mg intestinal alkaline phosphatase/kg of diet; 30 IAP: diet with the addition of 30 mg intestinal alkaline phosphatase/kg of diet.
[2]SEM: standard error of the mean.
[3]Significance level.

Table 3. Additional dietary effect of intestinal alkaline phosphatase on the pH of digestive tract contents of piglets challenged with *Escherichia coli* $K88^+$.

Items	Experimental treatments[1]				SEM[2]	*P-value*[3]
	Control	AGP	15 IAP	30 IAP		
pH of digestive tract contents						
Stomach	3.706	3.063	3.361	3.538	0.133	0.370
Jejunum	5.608	5.905	5.983	5.531	0.121	0.476
Cecum	5.378	5.333	5.363	5.453	0.042	0.597
Colon	5.611	5.796	5.641	5.826	0.047	0.289

[1]Experimental diets - Control: diet without feed additive; AGP: diet with the addition of antimicrobial growth promoter; IAP: diet with the addition of 15 mg intestinal alkaline phosphatase/kg of diet; 30 IAP: diet with the addition of 30 mg intestinal alkaline phosphatase/kg of diet.
[2]SEM: standard error of the mean.
[3]Significance level.

Table 4. Additional dietary effect of intestinal alkaline phosphatase on the histopathological description of the jejunum of piglets challenged with *Escherichia coli* K88[+].

Items	Experimental treatments[1]				*P-value*[2]
	Control	AGP	15 IAP	30 IAP	
Cell infiltrate	1.166	0.416	0.666	0.416	0.200
Epithelial hyperemia	1.416	1.166	1.416	1.083	0.101
Epithelial desquamation	1.583	0.833	1.166	1.166	0.174
Coccidiosis	0	0	0	0	-
Bacterial lumps	0.666	0	0.166	0	0.130
Rods	0	0	0	0	-
Cysts	0	0	0	0	-
Mucus	0.166	0	0.166	0	0.428
Goblet cells	1.916	2.166	2.250	2.000	0.400
Tissue necrosis	0.250	0.500	0.166	0.083	0.086

[1]Experimental diets - Control: diet without feed additive; AGP: diet with the addition of antimicrobial growth promoter; IAP: diet with the addition of 15 mg intestinal alkaline phosphatase/kg of diet; 30 IAP: diet with the addition of 30 mg intestinal alkaline phosphatase/kg of diet.
[2]Significance level of observed averages.
[3]Histopathological description – 0 = absent; 1 = discrete; 2 = moderate; 3 = intense.

Table 5. Additional dietary effect of intestinal alkaline phosphatase on the tumor 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 challenged with *Escherichia coli* K88^{+}.

Items	Experimental treatments[1]				SEM[2]	*P-value*[3]
	Control	AGP	15 IAP	30 IAP		
Jejunum						
TNF-α (μm²)	0.250	0.1806	0.084	0.060	0.044	0.454
COX-2 (μm²)	12.599	15.712	6.707	14.703	1.657	0.285
TLR4 (%)	12.33	14.206	15.558	10.615	1.165	0.319
PCNA (%)	10.467	7.720	8.800	9.383	0.811	0.668
Liver						
TNF-α (μm²)	3.482	4.892	4.084	2.571	0.381	0.217
COX-2 (μm²)	16.970	14.665	11.312	17.571	1.639	0.624
TLR4 (%)	19.708	15.594	11.048	20.785	2.016	0.243
PCNA (%)	8.733	15.367	15.500	14.900	1.211	0.127
Glycogen reserve (%)	18.49	31.207	37.657	29.312	3.251	0.236

[1]Experimental diets - Control: diet without feed additive; AGP: diet with the addition of antimicrobial growth promoter; IAP: diet with the addition of 15 mg intestinal alkaline phosphatase/kg of diet; 30 IAP: diet with the addition of 30 mg intestinal alkaline phosphatase/kg of diet.
[2]SEM: standard error of the mean.
[3]Significance level.