https://doi.org/10.17221/82/2021-CJAS

Biological response of piglets challenged with *Escherichia coli* F4 (K88) when fed diets containing intestinal alkaline phosphatase

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Citation: Genova J.L., Rupolo P.E., Melo A.D.B., Santos L.B.A., Wendt G.N., Barbosa K.A., Carvalho S.T., Oliveira N.T.E., Costa L.B., Carvalho P.L.O. (2021): Biological response of piglets challenged with *Escherichia coli* F4 (K88) when fed diets containing intestinal alkaline phosphatase. Czech J. Anim. Sci., 66: 391–402.

Abstract: The aim of the study was to investigate the effect of intestinal alkaline phosphatase (IAP) added to diets on growth performance, diarrhoea incidence (DI), blood metabolites, relative organ weight and intestinal morphometry of weaned piglets challenged with enterotoxigenic *Escherichia coli* F4 (K88). A total of 64 crossbred entire male piglets (25-day-old and 7.16 ± 0.28 kg body weight) were allocated into four treatments: control diet (CD^-), CD^- + antimicrobial growth promoter (AGP), CD^- + 15 mg IAP/kg of diet and CD^- + 30 mg IAP/kg of diet, with eight replications. At 15 days, all piglets were orally challenged with 6 ml of a solution containing K88 (10^6 colony forming units/ml). Microencapsulated IAP in acid solution showed 14.43% solubility and pH values of 1.69, 1.72, 1.51, and 1.52 at the different times measured (0.5 h, 1.0 h, 17.0 h and 24 h); differently, IAP in basic solution had 4.10% solubility and pH values increased (5.95, 6.10, 6.32 and 6.63) according to the different times, respectively. On days 25–35, piglets that received 30 mg IAP and CD^- showed better feed conversion ratio ($P = 0.075$) compared to those fed 15 mg IAP. Piglets that consumed 30 mg IAP or CD^- had higher ($P = 0.004$) average daily gain on days 35–44. On days 35–44, piglet average daily feed intake was lower ($P = 0.033$) with 15 mg IAP compared to AGP. In the entire period, piglets fed 15 mg IAP showed a reduction in average daily gain ($P = 0.040$) and average daily feed intake ($P = 0.092$). Piglets on 30 mg IAP showed an improvement ($P \leq 0.05$) in DI in the pre- and post-challenge period. The relative spleen weight of the piglet increased ($P = 0.043$) in response to 30 mg IAP. Overall, the addition of 30 mg IAP to diets improves the growth performance, attenuates DI and promotes an increase in spleen relative weight to maintain the health state in piglets.

Keywords: antimicrobial growth promoter; growth performance; post-weaning diarrhoea; weanling piglet

Supported by the National Council for Scientific and Technological Development (CNPq, Project No. 449220/2014-1).

https://doi.org/10.17221/82/2021-CJAS

The development of the gastrointestinal tract of piglets in the post-weaning period is a highly specialised, dynamic and constantly changing process (Pluske 2016). 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 and is closely associated with growth performance analysis (Sun and Kim 2017).

Currently, 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 feeds and management practices that influence or intend to influence different aspects of intestinal health and mitigate negative effects from abrupt weaning (Jayaraman and Nyachoti 2017).

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, mainly including F4 $(K88)^+$ and $F18^+$ (Sun and Kim 2017). Thus, it is necessary to search for new safe and effective solutions to minimise losses caused by bacterial infections. 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 the inflammatory activity of TNF-α levels (Moss et al. 2013), protection against diarrhoea and other enteric infections (Alam et al. 2014) and the ability to promote bacterial growth and normalise the intestinal microbiome (Malo et al. 2014).

Therefore, the aim of this study was to investigate the effect of intestinal alkaline phosphatase added to diets on the growth performance, diarrhoea incidence, blood metabolites, intestinal morphometry and relative organ weight of weaned piglet challenged with *E. coli* F4 (K88), and the *in vitro* simulation of microencapsulated IAP on the pH modulation capacity and its dilution in acidic or basic solution.

MATERIAL AND METHODS

The experiment was carried out in the Pig 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).

Experimental design, animals, housing and diets

A total of 64 crossbred piglets (Landrace × Large White, Agroceres♂ and DanBred♀), entire male weaned at 25 days of age with an average initial body weight of 7.16 ± 0.28 kg were assigned to a randomised complete block design consisting of four treatments and eight replications, totalling 32 experimental units (EU), with two animals per EU. The blocks were based on initial body weight.

At the beginning of the experimental period, the animals were weighed and identified with numbered ear tags and housed in a masonry nursery facility and ceramic roof tiles, consisting of suspended pens (1.54 m^2), with polyethylene plastic flooring, equipped with nipple-type drinking fountains and gutter-type feeders, arranged in two rows, divided by a central aisle, where they remained for a period of 19 days.

Ambient temperature and relative humidity were recorded using a data logger with a digital display, which was installed in the centre of the experimental building. The minimum recorded temperature of the internal environment was 19.1 ± 5.2 °C, and the maximum was 29.7 ± 5.5 °C. The nursery facility was ventilated with fans, exhaust fans and tilting-type windows. The heating of the experimental pens was controlled using individual infrared incandescent lamps.

The diets were formulated to meet the piglet requirements for pre-starter growth phase I and II, following the nutritional requirements proposed by Rostagno et al. (2017). The experimental treatments (Table 1) were composed of control diet (negative control), control diet + antimicrobial growth promoter (AGP, 150 mg tiamulin/kg of diet) as a positive control, control diet + 15 mg IAP (P7640, type I-S obtained from bovine intestinal mucosa, lyophilised powder, ≥ 10 diethanolamine units/mg solid which hydrolyses 1.0 micromole of *p*-nitrophenyl phosphate per min at pH 9.8 at 37 °C; Sigma-

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Table 1. Ingredient composition and diets provided to piglets in the experimental period (%, as fed basis)

Items	Control		Control + AGP		Control + 15 IAP		Control + 30 IAP	
	PI	PII	PI	PII	PI	PII	PI	PII
Grain maize 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 soybean 43.16%	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[1]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Vitamin premix[1]	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
AGP[2]	–	–	0.015	0.015	–	–	–	–
Microencapsulated IAP[3]	–	–	–	–	2.00	2.00	4.00	4.00
Calculated values								
Crude protein (%)	21.42	19.87	21.42	19.87	21.42	19.87	21.42	19.87
Metabolisable energy (Mcal/kg)	14.24	14.13	14.24	14.13	14.24	14.13	14.24	14.13
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

15 IAP = 15 mg intestinal alkaline phosphatase/kg of diet; 30 IAP = 30 mg intestinal alkaline phosphatase/kg of diet; AGP = antimicrobial growth promoter; PI = pre-starter I; PII = pre-starter II

[1]Nutritional levels per kg of premix, (mg/kg): Mn sulphate (120), Zn oxide (160), Fe sulphate (120), Cu sulphate (20), I (2), sodium selenite (1.2), 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); (μg/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); [2]tiamulin: 150 mg/kg of diet; [3]intestinal alkaline phosphatase type I-S obtained from bovine intestinal mucosa (Sigma-Aldrich, Inc., St, Louis, MO, USA)

Aldrich, Inc., St, Louis, MO, USA)/kg of diet and control diet + 30 mg IAP/kg of diet.

Microencapsulation of intestinal alkaline phosphatase

The microencapsulation process was carried out by the Palsgaard Candon Company and consisted of the dilution of the enzyme in rice starch (RS), propylene glycol ester (PGE) + palmitic acid (PA) at the ratio of 1 g/1.333 kg. The final composition of the coating material used in the microencapsulation process presented the following proportions: 50% RS, 30% PGE + 20% PA.

Intestinal alkaline phosphatase was microencapsulated in 50% RS + 50% PGE (IAP I) or microencapsulated in 50% RS + 50% PGE with PA (IAP II).

https://doi.org/10.17221/82/2021-CJAS

The procedures tested were IAP in the forms: pure lyophilised (IAP), IAP I and IAP II induced or not with *E. coli* LPS. As control procedures were used alveolar macrophage (AM), AM + phosphate-buffered saline (PBS) and AM + IAP groups, without the addition of lipopolysaccharide (LPS). Macrophages were isolated by bronchoalveolar lavage (BAL) from a mare. In the laboratory, the BAL fluid was centrifuged and the pellet obtained was suspended in the animal's own supernatant.

From the cell pellet, 10 µl were used for slide mounting and differential cell count in 1 000× magnification. After counting, the cell suspension was adjusted to 5×10^6 viable cells/ml and 100 µl were plated into 96-well microplate wells. After the isolation of the AM, the phosphate-buffered saline (PBS, control group) and ETEC LPS (100 ng/ml) inflammatory inducers were added in the amount of 50 µl. Plates were incubated for 60 min 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.

The resulting solution had its absorbance read in a spectrophotometer with a wavelength of 550 nm. To analyse the phagocytic activity of AM, after the macrophage plates had their wells washed twice with PBS, 100 µl PBS and 20 µl zymosan were added to each well, stained with neutral red and incubated at 37 °C for 30 minutes. Then, the supernatant was discarded, and the cells were fixed with Baker's solution for 30 min at 37 °C. The supernatant was discarded, and 100 µl PBS was added to perform centrifugation at 400 *g* for 5 minutes. Then, the neutral red dye was solubilised with 200 µl extraction solution and incubated for 30 minutes. After this period, the absorbance was measured at 550 nm. The final result of adhesion and phagocytic activity was corrected by the percentage of alveolar macrophages in the BAL fluid (absorbance × % macrophages).

Solubility and pH change of post-microencapsulation IAP

In vitro solubility was determined by the percentage of microencapsulated IAP weight loss and pH change. A sample amount (15 ± 0.00 g) was weighed into identified and pre-weighed Erlenmeyer flask (n = 8). Subsequently, 100 ml of acidic solution (0.1 mol HCl) or basic solution (0.5% sodium dodecyl sulphate) were added, sealed with aluminium foil and then placed in a water bath at 37 °C for 24 h. At each time interval (0.5 h, 1 h, 17 h and 24 h), pH measurement and agitation of the flasks for 1 min were performed. After 24 h, the solution with the microencapsulated IAP was filtered through pre-weighed quantitative filter paper, washing the remainder with distilled water. The retained portion 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"). Solubility was expressed as a percentage of weight loss.

Growth performance testing and diarrhoea incidence

The animals received feed and water *ad libitum* throughout the experimental period. Performance variables were determined on days 25–35, 35–44, 40–44 and 25–44 of piglet age, through the bodyweight of the animals and the quantification of the feed provided and wasted on the floor and feeder in each pen. Based on these data, average daily feed intake (kg/day), average daily gain (kg/day), and feed conversion ratio (kg/kg) were calculated. The diarrhoea incidence was recorded daily at 9:00 a.m. before cleaning the experimental unit. The presence or absence of diarrhoea (liquid faeces on the floor and/or dirty anal region) was calculated as the proportion of animals with diarrhoea in each experimental phase.

Blood sampling

In order to evaluate the blood metabolite concentration, piglets were kept on an 8-h fasting diet at the end of each experimental phase. Then, blood collection (± 10 ml) was performed at 8:30 a.m. via puncture of the anterior cranial vena cava of 32 animals using 0.7 × 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 (UNIOESTE). The blood samples were centrifuged at 3 000 *g* for 10 minutes. Approximately three ml of each tube

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were then transferred to previously identified and frozen Eppendorf polyethylene tubes for the analysis of urea (enzymatic-colorimetric method), glucose (enzymatic-colorimetric method) and alkaline phosphatase (kinetic-colorimetric method). These analyses were determined by spectrophotometry using specific Gold Analytical Diagnostic kits (Gold Analisa, Belo Horizonte, Brazil).

Bacterial strain and challenge procedure

On day 15 of experimentation (day of infection), piglets were subjected to an 8-h fasting and were challenged individually, receiving 6 ml of bacterial suspension containing a dose of 10^6 colony forming units (CFU)/ml of an ETEC F4 strain (K88), isolated from weanling pig faeces (post-weaned 21-days old) and provided by Mercolab (Cascavel, Paraná, Brazil). The bacteria were seeded in plates containing eosin methylene blue medium and incubated at 36 ± 1 °C for 48 hours. Colonies of ETEC F4 strain presented a characteristic green metallised reflex due to the rapid lactose fermentation. A single colony from each plate was collected and spread onto brain heart infusion agar and incubated at 36 ± 1 °C for 24 h until the concentration of 1.0×10^9 CFU/ml was reached. Subsequently, serial dilutions were performed in saline solution (0.9% NaCl) to reach the concentration of 1.0×10^6 CFU/ml. The infection of 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 inoculants passage into the lungs was minimised.

Relative organ weight (%) and sampling of the intestinal epithelium

On the 19th day of experimentation, six animals from each experimental treatment were slaughtered (after 6-h fasting) following humane slaughter methods. The choice of the animal to be slaughtered was according to bodyweight nearest to the average treatment. The digestive and non-digestive organs were removed, washed with distilled water and weighed, and the length of the animal's small bowel was measured. Relative organ weight (%) was calculated considering the animal's body weight at the time of slaughter.

Segments of the approximately 3-cm length of the jejunum were collected (Guo et al. 2001), washed with physiological solution (0.9% NaCl) and stored in 50 ml sterile plastic pots containing 10% buffered formaldehyde solution for 48 hours. Subsequently, the samples were sent to the Histopathology Laboratory of Mercolab where they were paraffin-embedded and microtomized for slide mounting. The slides were stained with haematoxylin and eosin for histological description.

Statistical analyses

The standardised residuals analysis of Student (RSTUDENT) was performed in order to identify outliers (values greater than or equal to three standard deviations). For the growth performance, blood metabolites and relative organ weight, the statistical model was fitted with treatment as a fixed effect, block as random, and covariate effect. 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 diarrhoea incidence (DI) the data were transformed into binary values, being presented as percentage results. For the DI, the generalised linear model was fitted to the data using the binomial distribution and logit link function. The DI was compared using a test of the difference between the least-squares means, through the χ^2 statistic. Comparisons between treatment averages were performed according to Tukey's post hoc test. The β error of the growth performance parameters was used ($0.05 < P < 0.1$). The other variables were analysed using the general linear models procedures of the statistical software SAS University Edition (SAS Inst. Inc., Cary, NC, USA).

RESULTS

Process of evaluation of post-microencapsulation IAP activity and solubility and *in vitro* pH modulation capacity for 24 h

It was observed that macrophage adhesion was higher for treatments composed of AM + LPS + IAP I and AM + LPS + IAP II. Phagocytic activity related to macrophage adhesion was lower

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for the groups AM + LPS + IAP, AM + LPS + IAP I and AM + LPS + IAP II compared to the other treatments. However, the group with AM + LPS + IAP II presented the lowest phagocytic activity. After the pilot analysis of the phagocytic 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, IAP in acid solution showed 14.43% solubility and pH values of 1.69, 1.72, 1.51, and 1.52 at the different times measured (0.5 h, 1.0 h, 17.0 h and 24 h). Differently, IAP in basic solution had 4.10% solubility; however, pH values increased (5.95, 6.10, 6.32 and 6.63) according to the different times, respectively.

Growth performance testing and diarrhoea incidence

Prior to the growth performance test, none of the animals showed clinical signs of post-weaning diarrhoea. IAP has the ability to improve the growth performance of piglets in pre-starter phase II and in the entire 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 deter-

Table 2. Effect of intestinal alkaline phosphatase on the growth performance of piglets challenged with *Escherichia coli* F4 (K88)

Items	Experimental treatments				SEM	*P*-value	1-β (%)[1]
	control	AGP	15 IAP	30 IAP			
Pre-starter phase I (7.168 kg to 8.893 kg) – 25 to 35 days of age							
IBW (kg)	7.17	7.16	7.16	7.16	0.050	–	–
FBW (kg)	9.10	8.85	8.80	8.81	0.105	0.450	22.783
ADG (kg)	0.19	0.16	0.16	0.16	0.009	0.450	22.252
ADFI (kg)	0.24	0.23	0.22	0.21	0.010	0.282	32.886
FCR (kg/kg)	1.29^{b}	1.48^{ab}	1.50^{a}	1.28^{b}	0.045	0.075	57.071
Pre-starter phase II (8.893 kg to 11.191 kg) – 35 to 44 days of age							
FBW (kg)	11.53	11.26	10.70	11.26	0.190	0.380	25.818
ADG (kg)	0.27^{a}	0.24^{ab}	0.17^{b}	0.27^{a}	0.011	0.004	90.208
ADFI (kg)	0.37^{ab}	0.38^{a}	0.29^{b}	0.36^{ab}	0.014	0.033	70.396
FCR (kg/kg)	1.41	1.37	1.61	1.39	0.062	0.359	26.793
Post-challenge phase (9.428 kg to 11.191 kg) – 40 to 44 days of age							
IBW (kg)	9.86	9.56	8.84	9.44	0.200	–	–
FBW (kg)	11.53	11.26	10.70	11.26	0.190	0.380	25.818
ADG (kg)	0.38	0.38	0.30	0.39	0.017	0.216	37.612
ADFI (kg)	0.39	0.41	0.32	0.39	0.016	0.163	42.202
FCR (kg/kg)	1.00	1.06	1.12	1.01	0.037	0.615	15.947
Total period (7.168 kg to 11.191 kg) – 25 to 44 days of age							
FBW (kg)	11.53	11.26	10.70	11.26	0.190	0.380	25.818
ADG (kg)	0.23^{a}	0.20^{ab}	0.17^{b}	0.21^{ab}	0.008	0.040	68.176
ADFI (kg)	0.31^{a}	0.31^{a}	0.25^{b}	0.28^{ab}	0.011	0.092	55.387
FCR (kg/kg)	1.35	1.41	1.51	1.33	0.042	0.382	25.597

15 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; ADG = average daily gain; ADFI = average daily feed intake; AGP = antimicrobial growth promoter; ANT = diet with the addition of antibiotic; control = diet without feed additive; FBW = final body weight; FCR = feed conversion ratio; IBW = initial body weight

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

[a,b]Averages followed by different lowercase letters in the row differ according to Tukey's test at 5% and 10% probability

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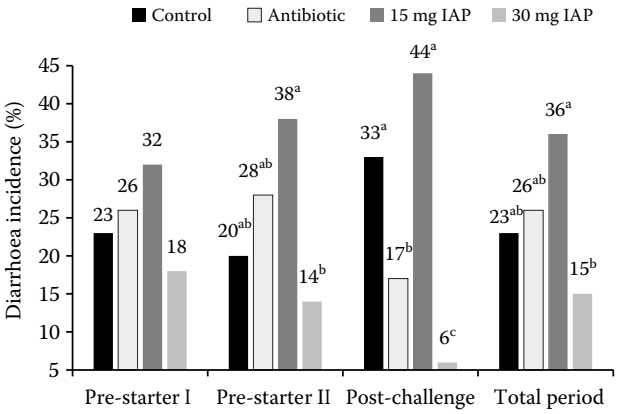

Figure 1. Effect of intestinal alkaline phosphatase on diarrhoea incidence in piglets challenged with *Escherichia coli* F4 (K88)

Diarrhoea incidence was calculated as the proportion of animals with diarrhoea

[a–c]Observed proportions of diarrhoea incidence, followed by different lowercase letters, differ from each other by testing the difference between the lsmeans at the 5% probability level

mined. Before the challenge with ETEC, in pre-starter phase I, piglets that received 30 mg of IAP added to the diet or control group showed a better feed conversion ratio (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 gain in pre-starter phase II. Piglets fed 15 mg of IAP had lower average daily feed intake (P = 0.033) compared to piglets with diets containing AGP. When we evaluated the effect of IAP over the entire period, there was a difference between treatments, in which the piglets fed 15 mg of IAP showed a reduction in average daily gain (P = 0.040) and average daily feed intake (P = 0.092) (Table 2).

There was no effect (P > 0.05) of treatments on DI in pre-starter phase I. However, piglets fed 30 mg IAP showed a reduction (P = 0.044) in DI when compared to those that received 15 mg IAP in pre-starter phase II. This effect was maintained for the entire period (P = 0.007). The key result was observed (P = 0.009) in the post-challenge phase, in which piglets receiving 30 mg IAP showed lower DI compared to the other treatments (Figure 1).

Blood metabolites

No differences (P > 0.05) between treatments were obtained in any of the pre- and post-challenge plasma concentration indicators (Table 3).

Table 3. Effect of intestinal alkaline phosphatase on blood metabolites of piglets challenged with *Escherichia coli* F4 (K88)

Items	Experimental treatments				SEM	*P*-value
	control	AGP	15 IAP	30 IAP		
Initial average plasma concentrations (baseline)						
Alkaline phosphatase (IU/l)	494.42	557.70	594.41	455.62	122.937	–
Urea (mg/dl)	23.01	18.68	19.66	19.44	1.008	–
Glucose (mg/dl)	120.57	116.80	111.98	110.70	4.757	–
Pre-starter phase I (7.168 kg to 8.893 kg) – 25 to 35 days of age						
Alkaline phosphatase (IU/l)	441.60	666.40	346.60	365.50	65.219	0.172
Urea (mg/dl)	21.52	24.11	24.72	20.02	1.150	0.477
Glucose (mg/dl)	118.50	128.66	114.41	122.26	3.159	0.421
Pre-challenge phase (8.893 kg to 9.428 kg) – 35 to 40 days of age						
Alkaline phosphatase (IU/l)	633.00	598.00	386.90	407.20	56.224	0.445
Urea (mg/dl)	23.57	23.04	25.30	25.28	2.040	0.849
Glucose (mg/dl)	97.98	97.34	88.63	93.62	4.314	0.740
Post-challenge phase (9.428 kg to 11.191 kg) – 40 to 44 days of age						
Alkaline phosphatase (IU/l)	317.60	473.10	425.90	344.10	41.881	0.609
Urea (mg/dl)	18.42	19.86	22.58	21.73	1.009	0.569
Glucose (mg/dl)	89.51	99.01	91.01	89.49	3.305	0.696

15 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; AGP = antimicrobial growth promoter; control = diet without feed additive

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Relative organ weight (%) and intestinal morphometry

To determine whether IAP also affects the relative organ weight, digestive and non-digestive organs were evaluated. Piglets that received a 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 4). There was no effect (P > 0.05) of treatments on the intestinal morphometry of piglets; however, there was a slight increase (P = 0.106) in the VH : CD ratio of piglets when fed 30 mg of IAP (Table 4).

DISCUSSION

Process of evaluation of post-microencapsulation IAP activity and solubility and *in vitro* pH modulation capacity for 24 h

By reducing LPS-induced phagocytic activity, it is suggested that IAP reduced the toxic effect of LPS, since the TLR4 and IAP expression increased in the presence of LPS (Chen et al. 2010). This result is associated with the IAP 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 higher 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 coating material in the microencapsulation process. In relation to pH oscillation, these results may be related to the absence of buffers in the solutions, which influenced the pH variations, and not due to the effect of IAP ability to modulate the pH because the solubility was higher in the acidic solution and under these medium conditions there is neither IAP activity nor pH modulation capacity (Koyama et al. 2002). 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).

Growth performance testing and diarrhoea incidence

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 post-

Table 4. Effect of intestinal alkaline phosphatase on the relative weight of digestive and non-digestive organs (%) and intestinal morphometry of piglets challenged with *Escherichia coli* F4 (K88)

Items	Experimental treatments				SEM	*P*-value
	control	AGP	15 IAP	30 IAP		
Empty stomach	0.81	0.71	0.81	0.77	0.019	0.230
Empty small bowel + pancreas	4.88	5.05	5.21	4.63	0.137	0.661
Empty caecum	0.23	0.23	0.28	0.25	0.010	0.403
Empty colon	1.97	2.30	2.01	2.01	0.064	0.329
Liver + gallbladder	3.15	3.35	3.22	2.93	0.076	0.148
Spleen	0.18^{b}	0.19^{ab}	0.20^{ab}	0.23^{a}	0.006	0.043
Kidneys	0.54	0.55	0.51	0.52	0.012	0.647
Small bowel (m)	10.18	9.75	10.64	9.29	0.269	0.305
Intestinal morphometry (μm)						
Villus height (VH)	398.87	374.17	377.75	424.83	0.013	0.582
Crypt depth (CD)	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	0.106

15 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; AGP = antimicrobial growth promoter; control = diet without feed additive
^{a,b}Averages followed by different lowercase letters in the row differ according to Tukey's test at 5% probability

https://doi.org/10.17221/82/2021-CJAS

weaning diarrhoea. Alam et al. (2014) considered antibiotics as an additive that inherently causes dysbiosis, an imbalance in the number and composition of commensal intestinal bacteria, which eventually did not occur in piglets in the control group or those that consumed 30 mg of IAP. Study reports also showed that treatment with tiamulin as an AGP triggered intestinal dysbiosis (Le Roy et al. 2019; Saettone et al. 2020).

These results are supported by the explanations that IAP added at 30 mg acted as a host defence 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, it improves the growth performance of piglets. In this first trial with piglets, it was found that the addition of 15 mg of IAP to the diet was not sufficient to attenuate the critical post-weaning period. We speculate that the reason why piglets that consumed 15 mg of IAP showed no response to performance 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 the small intestine.

Based on previous experimental reports (Alam et al. 2014; Malo et al. 2014), IAP is a defence factor of the intestinal mucosa, a local immunomodulator (Chen et al. 2011), which positively influences the piglet growth performance, demonstrating the favourable effect of IAP in reducing the intestinal inflammatory response (Lackeyram et al. 2010). However, the piglets in the negative control group also showed a favourable response and the reason for this inconsistency is that the efficiency of each additive depends on the diet (composition, feed processing, feeding methods), colonisation and associated succession of microbial populations, stress and genetics (Celi et al. 2017).

This can be attributed to the fact that the diet without feed additives was able to promote an intestinal health status and beneficially influence the resident intestinal microbiota without affecting the growth performance variables. In this study, the animals were housed in previously disinfected experimental facilities, and these sanitary conditions may have reduced environmental contamination. In addition, the short experimental challenge period and complex and highly digestible diets used in the present study were unlikely to cause impairment in animal performance. Alam et al. (2014) determined the efficacy of oral supplementation of IAP via drinking water in mice and observed positive results for protection against DI and enteric infections. However, the variation that occurs in the responses of piglets that consumed feed additives is presumably a consequence, in part, of many different management conditions they are exposed to, the amount added to the diet and differences in age, health status or environmental conditions (Liu et al. 2018).

IAP activity in the prevention of DI has been confirmed (Alam et al. 2014). The effects of IAP are based on its ability to rapidly restore commensal intestinal microbiota in studies involving mice (Chen et al. 2011; Moss et al. 2013) and piglets (Beumer et al. 2003). Piglets challenged with ETEC exhibited a considerably greater DI because ETEC infection is associated with an impaired intestinal barrier function (Yang et al. 2014), and the F4 inoculation significantly increased DI in piglets that did not consume 30 mg of IAP. These results are explained by the role of inhibiting adhesion and bacterial internalisation, preventing disruption of the barrier integrity (Song et al. 2015).

Blood metabolites

Notably, urea and glucose concentrations are close to the established range for the species studied (Nielsen et al. 2015; 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).

An increased blood urea concentration also occurs due to high protein catabolism and stress. In this sense, 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, age, gender, breed, nutrition and health status (Klem et al. 2010), 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).

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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 challenge or show acute clinical signs of disease may undergo a decrease in glucose concentration 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 hyperinsulinaemic (Nielsen et al. 2015).

The plasma ALP concentration has a wide oscillation, being regulated by several factors such as diet (Zhang et al. 2018), stress (Mayengbam and Tolenkhomba 2015), body growth rate, age and genetics (Abeni et al. 2018); 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 post-weaning and/or challenge period. The antibiotics addition to diets has a metabolic effect, the increase in the IAP response as a way to restore the intestinal microbiota in the dysbiosis condition.

Relative organ weight (%) and intestinal morphometry

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 the greater immune system stimulation. The effect of IAP is evident, 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 (Mussa et al. 2013) and recruit defence cells (Beutler and Rietschel 2003), which influences 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, which apparently demonstrates a normal state of organ development. However, it was not evaluated by immunohistopathological analysis (Pluske 2016). 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; Malo et al. 2014). The toxicity generated by the challenge with ETEC has characteristic histopathology in mammals, including liver, heart and intestinal lesions, as well as oedema in the affected tissues, which may promote the exacerbated organ growth (Bates et al. 2007), which did not occur in the present study.

Intestinal histology was examined, and a villus height (VH) reduction was associated with post-weaning growth lag and reduction in average daily feed intake. However, the dietary treatments did not affect the intestinal morphometry variables in the study, and there is no explanation for these results, since the increase in DI and lower growth performance are related to reduced VH (Rong et al. 2015). On the other hand, the capacity to absorb nutrients with lower energy losses due to the cell turnover is improved by greater VH and lower CD, i.e. higher VH : CD ratio (Owusu-Asiedu et al. 2003).

No intestinal damage as a result of ETEC infection was observed in terms of intestinal morphometry in the present study, but the VH : CD ratio is a useful criterion for assessing intestinal health and function (Pluske 2016).

Based on the evaluation criteria of the present study, the addition of 30 mg IAP to diets promoted improvements in the growth performance and reduced the diarrhoea incidence, which can be considered a potentially effective alternative in replacement of AGP in diets for weaned piglets. In addition, the higher dose of IAP promotes an increase in spleen relative weight to maintain the health state without altering blood metabolites and intestinal morphometry in piglets challenged with enterotoxigenic *E. coli* F4 (K88).

Acknowledgements

We are grateful for support to the Palsgaard Candon Company (method of microencapsulation), Copagril Company (ingredient and animal supply), Laboratory of the Mercolab (bacterial strain), State University of Western Paraná (PPZ, Marechal Cândido Rondon, Brazil), Pontifical Catholic University of Paraná (School of Life Sciences, Curitiba, Brazil), Copisces Company (ingredient supply) and Carboni Company (ingredient supply).

https://doi.org/10.17221/82/2021-CJAS

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

Abeni F, Petrera F, Dal Pra A, Rapetti L, Crovetto GM, Galassi G. Blood parameters in fattening pigs from two genetic types fed diet with three different protein concentrations. Transl Anim Sci. 2018 Oct;2(4):372-82.

Alam SN, Yammine H, Moaven O, Ahmed R, Moss AK, Biswas B, Ghosh S. Intestinal alkaline phosphatase prevents antibiotic-induced susceptibility to enteric pathogens. Ann Surg. 2014 Apr 1;259(4):715-22.

Bates JM, Akerlund J, Mittge E, Guillemin K. Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota. Cell Host Microbe. 2007 Dec 13;2(6):371-82.

Beumer C, Wulferink M, Raaben W, Fiechter D, Brands R, Seinen W. Calf intestinal alkaline phosphatase, a novel therapeutic drug for lipopolysaccharide (LPS)-mediated diseases, attenuates LPS toxicity in mice and piglets. J Pharmacol Exp Ther. 2003 Nov 1;307(2):737-44.

Beutler B, Rietschel ET. Innate immune sensing and its roots-the story of endotoxin. Nat Rev Immunol. 2003 Feb;3(2):169-76.

Brun LR, Brance ML, Lombarte M, Lupo M, Di Loreto VE, Rigalli A. Regulation of intestinal calcium absorption by luminal calcium content: Role of intestinal alkaline phosphatase. Mol Nutr Food Res. 2014 Jul;58(7):1546-51.

Celi P, Cowieson AJ, Fru-Nji F, Steinert RE, Kluenter AM, Verlhac V. Gastrointestinal functionality in animal nutrition and health: New opportunities for sustainable animal production. Anim Feed Sci Technol. 2017 Dec 1; 234:88-100.

Chen KT, Malo MS, Moss AK, Zeller S, Johnson P, Ebrahimi F, Mostafa G, Alam SN, Ramasamy S, Warren HS, Hohmann EL, Hodin RA. Identification of specific targets for the gut mucosal defense factor intestinal alkaline phosphatase. Am J Physiol Gastrointest Liver Physiol. 2010 Aug 1;299(2):G467-75.

Chen KT, Malo MS, Beasley-Topliffe LK, Poelstra K, Millan JL, Mostafa G, Alam SN, Ramasamy S, Warren HS, Hohmann EL, Hodin RA. A role for intestinal alkaline phosphatase in the maintenance of local gut immunity. Dig Dis Sci. 2011 Feb 21;56(4):1020-7.

Guo M, Hayes J, Cho KO, Parwani AV, Lucas LM, Saif LJ. Comparative pathogenesis of tissue culture-adapted and wild-type Cowden porcine enteric calicivirus (PEC) in gnotobiotic pigs and induction of diarrhea by intravenous inoculation of wild-type PEC. J Virol. 2001 Oct 1; 75(19):9239-51.

Jayaraman B, Nyachoti CM. Husbandry practices and gut health outcomes in weaned piglets: A review. Anim Nutr. 2017 Sep 1;3(3):205-11.

Klem TB, Bleken E, Morberg H, Thoresen SI, Framstad T. Hematologic and biochemical reference intervals for Norwegian crossbreed grower pigs. Vet Clin Pathol. 2010 Jun;39(2):221-6.

Koyama I, Matsunaga T, Harada T, Hokari S, Komoda T. Alkaline phosphatases reduce toxicity of lipopolysaccharides in vivo and in vitro through dephosphorylation. Clin Biochem. 2002 Sep 1;35(6):455-61.

Lackeyram D, Yang C, Archbold T, Swanson KC, Fan MZ. Early weaning reduces small intestinal alkaline phosphatase expression in pigs. J Nutr. 2010 Mar;140(3):461-8.

Le Roy CI, Woodward MJ, Ellis RJ, La Ragione RM, Claus SP. Antibiotic treatment triggers gut dysbiosis and modulates metabolism in a chicken model of gastro-intestinal infection. BMC Vet Res. 2019 Dec;15(1):1-13.

Liu Y, Espinosa CD, Abelilla JJ, Casas GA, Lagos LV, Lee SA, Kwon WB, Mathai JK, Navarro DMDL, Jaworski NW, Stein HH. Non-antibiotic feed additives in diets for pigs: A review. Anim Nutr. 2018 Jun 1;4(2):113-25.

Malo MS, Moaven O, Muhammad N, Biswas B, Alam SN, Economopoulos KP, Gul SS, Hamarneh SR, Malo NS, Teshager A, Mohamed MMR, Tao Q, Narisawa S, Millan JL, Hohmann EL, Shaw Warren H, Robson SC, Hodin RA. Intestinal alkaline phosphatase promotes gut bacterial growth by reducing the concentration of luminal nucleotide triphosphates. Am J Physiol Gastrointest Liver Physiol. 2014 May 15;306(10):826-38.

Mayengbam P, Tolenkhomba TC. Seasonal variation of hemato-biochemical parameters in indigenous pig: Zovawk of Mizoram. Vet World. 2015 Jun;8(6):732-7.

Moss AK, Hamarneh SR, Mohamed MMR, Ramasamy S, Yammine H, Patel P, Kaliannan K, Alam SN, Muhammad N, Moaven O, Teshager A, Malo NS, Narisawa S, Millan JL, Shaw Warren H, Hohmann E, Malo MS, Hodin RA. Intestinal alkaline phosphatase inhibits the proinflammatory nucleotide uridine diphosphate. Am J Physiol Gastrointest Liver Physiol. 2013 Mar 15;304 (6):G597-604.

Mussa T, Ballester M, Silva-Campa E, Baratelli M, Busquets N, Lecours MP, Dominguez J, Amadori M, Fraile L, Hernandez J, Montoya M. Swine, human or avian influenza viruses differentially activates porcine dendritic cells cytokine profile. Vet Immunol Immunopathol. 2013 Jul 15; 154(1-2):25-35.

Nielsen MF, Roelsgaard K, Keiding S, Brodersen K, Moller N, Vyberg M, Vilstrup H. Impaired hepatic counterregulatory

https://doi.org/10.17221/82/2021-CJAS

response to insulin-induced hypoglycemia in hepatic denervated pigs. J Clin Transl Endocrinol. 2015 Dec 1 25;2(4):131-6.

Owusu-Asiedu A, Nyachoti CM, Marquardt RR. Response of early-weaned pigs to an enterotoxigenic Escherichia coli (K88) challenge when fed diets containing spray-dried porcine plasma or pea protein isolate plus egg yolk antibody zinc oxide fumaric acid or antibiotic. J Anim Sci. 2003 Jul 1;81(7):1790-8.

Perri AM, O'Sullivan TL, Harding JC, Wood RD, Friendship RM. Hematology and biochemistry reference intervals for Ontario commercial nursing pigs close to the time of weaning. Can Vet J. 2017 Apr;58(4):371-6.

Pluske JR. Invited review: Aspects of gastrointestinal tract growth and maturation in the pre-and postweaning period of pigs. J Anim Sci. 2016 Sep 1;94(Suppl 3):399-411.

Rong Y, Lu Z, Zhang H, Zhang L, Song D, Wang Y. Effects of casein glycomacropeptide supplementation on growth performance intestinal morphology intestinal barrier permeability and inflammatory responses in Escherichia coli K88 challenged piglets. Anim Nutr. 2015 Jun 1;1(2):54-9.

Rostagno HS, Albino LFT, Donzele JL, Oliveira RF, Barreto SLT, Hannas MI, Sakamura NK, Perazzo FG, Saraiva A, Abreu MLT, Rodrigues PB, Brito CO. Brazilian Tables for poultry and swine: Food composition and nutritional requirements. 4th ed. Vicosa, Brazil: Universidade Federal de Vicosa; 2017. 488 p.

Saettone V, Biasato I, Radice E, Schiavone A, Bergero D, Meineri G. State-of-the-art of the nutritional alternatives to the use of antibiotics in humans and monogastric animals. Animals. 2020 Dec;10(12): 36 p.

Song ZH, Xiao K, Ke YL, Jiao LF, Hu CH. Zinc oxide influences mitogen-activated protein kinase and TGF-β1 signaling pathways, and enhances intestinal barrier integrity in weaned pigs. Innate Immun. 2015 May;21(4):341-8.

Sun Y, Kim SW. Intestinal challenge with enterotoxigenic Escherichia coli in pigs and nutritional intervention to prevent postweaning diarrhea. Anim Nutr. 2017 Dec 1; 3(4):322-30.

Yang KM, Jiang ZY, Zheng CT, Wang L, Yang XF. Effect of Lactobacillus plantarum on diarrhea and intestinal barrier function of young piglets challenged with enterotoxigenic Escherichia coli K88. J Anim Sci. 2014 Apr 1; 92(4):1496-503.

Zhang Y, Ward TL, Ji F, Peng C, Zhu L, Gong L, Dong B. Effects of zinc sources and levels of zinc amino acid complex on growth performance hematological and biochemical parameters in weanling pigs. Asian-Australas J Anim Sci. 2018 Aug;31(8):1267-74.

Received: May 14, 2021
Accepted: August 5, 2021
Published online: September 14, 2021