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Bone, microbiological and intestinal characteristics of piglets fed diets containing *Lithothamnium calcareum*

J. L. Genova[1#], N. T. E. Oliveira[1], C. P. Scherer [1], P. L. O. Carvalho[1], A. B. S. Costa[1], S. M. B. Artoni[2], A. C. G. Júnior[3], L. B. A. Santos[1], S. T. Carvalho[1] & J. Bortoluzzi[4]

[1]Animal Science Department, State University of Western Paraná, Marechal Cândido Rondon, PR 85960-000, Brazil

[2]Animal Science Department, Paulista State University, Jaboticabal, SP 14884-900, Brazil

[3]Agricultural Science Center - Agronomy, State University of Western Paraná, Marechal Cândido Rondon, PR 85960-000, Brazil

[4]Agricultural Science Center - Agronomy, Faculdade de Ensino Superior de São Miguel do Iguaçu Ltda/UNIGUAÇU, São Miguel do Iguaçu, PR 85877-000, Brazil

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Abstract

The aim of this study was to assess the effect of calcitic seaweed (CS), calcitic limestone (CL) and monodicalcium phosphate (DP) fed to piglets in various combinations on their gastrointestinal tract and bones. A total of 128 piglets (21 days old, 5.50 ± 0.49 kg bodyweight (BW) were assigned to treatments in a randomized complete block design and eight replications to one of four diets: D1: with CL + DP, D2: with CL, CS + DP, D3, similar to D2, but with 30% less calcium from CS and DP, and D4: with CS + DP. Treatments affected the metatarsal bones, mineral concentration in the heart and liver, and pH in the cecum and colon. Piglets fed D4 tended to have a greater *Enterobacteriaceae* count in the jejunum and ileum compared with D1 (4.82 vs 4.79 CFU/g). Piglets fed D1 and D3 had a greater Enterobacteriaceae count in their cecum than D4 (4.79 and 4.80 vs 4.76). The D2 and D1 treatments produced greater crypt depth (CD) in the duodenum and ileum compared with D3 and D4, respectively. Feeding D3 resulted in a greater villus height (VH) to CD ratio in the duodenum compared with D2 and showed a 24.5% increase in heart weight compared with fed D1. In conclusion, CS could be an alternative source of calcium source for piglets. The inconsistent findings of the present study suggest the need for further studies to better understand the interplay of effects of Ca^{2}+ source and level on its metabolism.

Keywords: bone density, calcitic limestone, intestinal microbiota, seaweed, weanling swine
[#]Corresponding author: jansllerg@gmail.com

Introduction

Diets composed of grains are deficient in Ca^{2+}, which is essential to animal nutrition. In addition, the Ca^{2+} in grains is insoluble because the formation of antinutrient complexes, which reduce its availability and absorption (Cowieson *et al.*, 2011). These circumstances make its supplementation necessary in the diet. Calcium is commonly supplemented in diets in the form of calcitic or dolomitic limestone and monocalcium or dicalcium phosphate, because these sources are low in cost. However, supplementation can lead to imbalances with other minerals, resulting in secondary deficiencies (González-Vega & Stein, 2016).

The use of piglets in studies of mineral nutrition has aroused interest because they can serve as a model organism for preclinical investigations with relevance to human nutrition (Knight & Dilger, 2018) In addition to their metabolic effects, minerals have a modulating effect on the intestinal microbiota (Blavi *et al.*, 2018). To reduce the use of inorganic Ca^{2+} and phosphorus (P) in diets for piglets, studies involving organic Ca^{2+} sources were performed with seaweed (Šimkus *et al.*, 2013; Adeleye & Blomfield, 2014; Gatrell *et al.*, 2014).

Lithothamnium calcareum is a limestone alga of the Corallinacea family, belonging to the red algae group (Almeida *et al.*, 2012). It is an organic source of Ca^{2+} and magnesium (Zhu *et al.*, 2014) and contains

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more than 20 trace elements that are bioavailable (Costa Neto *et al.*, 2010; Carlos *et al.*, 2011). Studies evaluating the use of calcitic seaweed include reports on Ca^{2+} digestibility similar to Ca^{2+} carbonate (González-Vega *et al.*, 2015) and similar effects on Ca^{2+} and P metabolism when compared with Ca^{2+} carbonate (Schlegel & Gutzwiller, 2017). However, it is not known whether *L. calcareum* influences the basic indicators of bone and gastrointestinal tract parameters of piglets in the nursery phase.

Therefore, the aim of this study was to assess the effect of CS, CL, and DP in various combinations on the relative weight and physicochemical composition of organs, bone structure, pH of contents of the gastrointestinal tract, microbial population, and intestinal morphometry of piglets in the nursery phase.

Materials and methods

The experiment was conducted in the Swine Sector of the Experimental Farm Professor Antonio Carlos dos Santos Pessoa of State University of Western Paraná (UNIOESTE), in Marechal Cândido Rondon, Brazil. All experimental procedures were approved by the UNIOESTE Research Ethics Committee (No. 14/16 - CEUA).

A total of 128 crossbred piglets, male entire of commercial lineage (Landrace x Large White, Agroceres♂ and DanBred♀) weaned at 21 days old with an average initial bodyweight (IBW) of 5.50 ± 0.49 kg were allocated to a randomized complete block design with four treatments, eight replications and four pigs per experimental unit (EU), totaling 32 EU (pens). The blocks were based on the IBW of the piglets.

At the beginning of the experiment, the animals were weighed and identified with numbered ear tags and housed in a nursery facility consisting of suspended pens (1.54 m²), with polyethylene plastic flooring, provided with nipple-type drinking and gutter-type feeders, arranged in two rows, divided by a central aisle. The experiment lasted 37 days.

The ambient temperature and relative humidity were recorded with a digital dial data logger (UNI-T UT 330B digital USB; Pequim, China). The minimum recorded temperature of the indoor environment was 22.00 ± 4.2 °C and the maximum was 25.00 ± 5.1 °C. The nursery facility was ventilated with fans, exhaust fan and tilting-type windows. The heating of the pens was controlled with individual infrared incandescent lamps per EU.

The diets were formulated to meet the piglets' requirements for the pre-starter I (6.01 to 9.42 kg), II (9.43 to 15.47 kg) and starter (15.48 to 22.88 kg) phases, following the nutritional requirements proposed by Rostagno *et al.* (2011), except for the treatment with 30% reduction in Ca^{2+} (Sakomura & Rostagno, 2007). The treatments were composed of four experimental diets: D1: with Ca^{2+} from CL + DP, D2: Ca^{2+} from CL + CS + DP, D3: 30% reduction in the Ca^{2+} supply from CS + DP (-30% Ca^{2+} diet), and D4: with Ca^{2+} from CS + DP (Table 1). Water and diets were provided ad libitum during the experiment. The animals did not receive antibiotic intervention.

At the end of the nursery experiment, eight animals from each treatment were slaughtered (after 6 hours solid-feed deprivation) following humane slaughter methods (electronarcosis followed by exsanguination). The selected animal was the one whose bodyweight was nearest to the average weight of the animals in its pen.

Immediately after slaughter, the pH of the stomach, jejunum, ileum, cecum, and colon contents was measured with a digital pH meter (model HI 99163, Hanna Instruments Inc., Rhodes Island, USAUSA) by inserting a unipolar electrode, adopting the methods described by Manzanilla *et al.* (2004) and Guo *et al.* (2001).

After pH measurement, the digestive organs (empty stomach, liver, and gallbladder, small intestine and pancreas, cecum, and colon) and non-digestive organs (spleen, heart, and kidneys) were removed, washed with saline solution (0.9% sodium chloride) and weighed (stainless steel digital scale, model UL50i; Pequim, China). The relative organ weights were then calculated, considering the animals' weight at slaughter.

The heart, liver and kidneys were pre-dried in a forced ventilation oven (Tecnal, SF-325 NM; Piracicaba, SP, Brazil) at 55 °C for 72 hours, then ground in a closed-chamber ball mill and stored in labelled flasks so that portions could be used to perform dry matter (DM) and mineral matter (MM) analyses. The mineral solutions (Ca^{2}+ and P) were obtained by digestion with nitroperchloric (4:1) and Ca^{2}+ and P concentrations of the organs were determined by reading inflame atomic absorption spectrophotometry (Tecnal brand, GBC 932 AA dual beam model; Dandenong, VIC, Australia) at the Agricultural Chemistry Laboratory and Environmental (UNIOESTE). The physicochemical analyses of the feeds and tissues were performed according to the methodologies described by AOAC (1990).

Table 1 Centesimal and chemical composition of diets for piglets in the nursery phase fed calcitic seaweed (%, as-fed basis)

Items	D1			D2			D3			D4		
	PI	PII	S	PI	PII	S	PI	PII	S	PI	PII	S
Corn grain, 7.88 % CP	51.70	53.05	66.20	51.66	52.98	66.12	51.87	53.41	66.43	51.59	52.89	66.02
Soybean meal, 45.22 % CP	8.06	17.91	23.86	8.00	17.88	23.84	8.00	17.75	23.70	8.06	17.86	23.80
Soybean oil	0.90	0.65	0.14	0.90	0.69	0.18	0.90	0.55	0.09	0.90	0.73	0.23
Fish meal, 54 % CP	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Whey protein concentrate, 34 % CP	21.15	13.46	-	21.15	13.46	-	21.15	13.46	-	21.15	13.46	-
Micronized soybean, 38 % CP	8.00	6.00	3.00	8.00	6.00	3.00	8.00	6.00	3.00	8.00	6.00	3.00
Calcitic limestone	0.57	0.62	0.70	0.31	0.34	0.38	-	-	-	-	-	-
Monodicalcium phosphate	1.21	1.08	1.20	1.21	1.08	1.21	0.86	0.77	0.86	1.21	1.08	1.21
Calcitic seaweed	-	-	-	0.31	0.31	0.38	0.80	0.80	0.99	0.68	0.74	0.83
Common salt	0.42	0.33	0.39	0.42	0.33	0.39	0.42	0.33	0.39	0.42	0.33	0.39
Sugar	4.00	3.00	-	4.00	3.00	-	4.00	3.00	-	4.00	3.00	-
Mineral and vitamin premix[1]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
L-lysine	0.10	0.04	0.61	0.10	0.05	0.61	0.10	0.05	0.61	0.10	0.05	0.61
DL-methionine	0.18	0.16	0.16	0.18	0.17	0.16	0.18	0.17	0.16	0.18	0.17	0.16
L-tryptophan	0.06	0.04	0.03	0.06	0.04	0.03	0.06	0.04	0.03	0.06	0.04	0.03
L-threonine	0.10	0.12	0.18	0.10	0.12	0.18	0.10	0.12	0.18	0.10	0.12	0.18
Calculated composition												
Crude protein, %	20.00	21.00	19.50	20.00	21.00	19.50	20.00	21.00	19.50	20.00	21.00	19.50
Total calcium, %	0.85	0.825	0.825	0.85	0.825	0.825	0.595	0.577	0.577	0.85	0.825	0.825
Metabolizable energy, mJ/kg	14.22	14.14	13.51	14.22	14.14	13.51	14.22	14.14	13.51	14.22	14.14	13.51
Available phosphorus, %	0.50	0.45	0.41	0.50	0.45	0.41	0.50	0.45	0.41	0.50	0.45	0.41

CP: crude protein, CL: calcitic limestone, DP: monodicalcium phosphate, CS: calcitic seaweed, (-30% Ca^{2+} diet): 30% reduction in the calcium supply.
D1: calcitic limestone + monodicalcium phosphate in diet, D2: calcitic limestone, calcitic seaweed and monodicalcium phosphate , D3: similar to D2, but with -30% less calcium, D4: calcitic seaweed and monodicalcium phosphate , PI: pre-starter I (6.01 to 9.42 kg), PII: pre-starter II (9.43 to 15.47 kg), S: starter (15.48 to 22.88 kg).
[1]Guaranteed levels per kg premix, folic acid 103.1, pantothenic acid 2250.0, ethoxyquin 206.0, biotin 16.9, iron sulfate 6733.4, vitamin B_1 225.0, vitamin B_2 537.5, vitamin B_6 437.5, vitamin K_3 375.0, sodium selenite 43.8, niacin 4687.5, manganese sulfate 1866.7, iodine 37.5, zinc oxide 1000.0, iodine 37.5, vitamin B_{12} 2537.5, chlorohydroxyquinoline 15.0, copper sulfate 22.1, lysine 123.8, methionine 110.3, threonine 46.6; and levels of vitamin D_3: 262500, vitamin E: 4250, vitamin A: 14375 IU/kg

After slaughter, content samples from the jejunum, ileum, cecum and colon were collected and destined for the counts of *Enterobacteriaceae* (Levine EMB) (Kasvi agar) and lactic acid bacteria (LAB) (MRS) (Acumedia agar) populations. The samples were stored in sterile plastic tubes, identified and transported under refrigeration for laboratory analysis according to Weedman *et al.* (2011).

To assess the structures of the intestinal epithelium, immediately after organ removal, segments approximately 3 cm length from the jejunum (extracted 150 cm from the ileocecal junction) and ileum (extracted 15 cm from the ileocecal junction) were harvested (Guo *et al.*, 2001). The slides were stained with hematoxylin and eosin for histological description (Gao *et al.*, 2000). Fifteen villi measurements and their crypts were analysed by sample.

The hind limbs collected during slaughter were cleaned with a scalpel and surgical scissors to remove the musculature and the third and fourth metatarsals without affecting bone tissue. Afterwards, the clean fresh bones were taken to the UNIOESTE's Animal Nutrition Laboratory for weighing (fresh weight) using an analytical scale and length measurement with a digital caliper (Lorben) with a measurement from 0.01 to 150 mm to calculate the Seedor index (Seedor *et al.*, 1991).

Dry matter was determined in a 105 °C oven (Tecnal TE 393/2, Piracicaba, SP, Brazil) and later the material was calcined in muffle furnace (Fornitec F2 DM single-phase, São Paulo, SP, Brazil) at 600 °C to obtain the MM. The mineral solutions (Ca^{2+} and P) obtained by digestion with nitroperchloric (3:1) were subjected to reading with atomic absorption equipment (Tecnal GBC 932 AA dual beam, Dandenong, VIC, Australia) at UNIOESTE to obtain the total mineral concentration.

Densitometry analysis of the metatarsal bones was performed in Hologic Discovery Wi® equipment, calibrated with a phantom. The bones were positioned in dorsoventral view for about three minutes. The reading was performed with the passage of two types of x-rays on the bone surface, with results on the bone mineral content (BMC) (g), bone area (cm^2), and bone mineral density (BMD) (g/cm^2).

For bone resistance to breaking, a three-point mechanical flexion test was performed with a universal mechanical testing machine (EMIC DL 10,000, with an EMIC load-cell of 200 kgf), following the standard (ANSI/ASAE S459 MAR 98) for the three-point flexion test with speed of 5 mm/min, 500 N preload with 30 seconds accommodation time and a distance between points of 60 mm.

The normality of experimental errors and the variance homogeneity between treatments for the characteristics had been evaluated with Shapiro-Wilk and Levine tests. Covariance analysis (ANCOVA) was performed to verify the effects of treatment class and IBW. When the IBW effect ($P \leq 0.05$) was detected, the statistical model was expressed by:

$$Y_{ijk} = m + T_i + b_j + \beta (X_{ijk} - \bar{X}_{...}) + \varepsilon_{ijk},$$

where: Y_{ijk} = average observation of the dependent variable in each plot, measured in the i-th class of feed, the j-th block and the k-th replication,
m = effect of the overall average,
T_i = effect of treatment classes, for i = (1, 2, 3, and 4),
b_j = effect of blocks, for j = (1, 2, 3, and 4),
β = regression coefficient of Y over X,
X_{ijk} = average observation of the covariate (IBW of the animal sampled in the pen) in each plot, measured in the i-th treatment class, the j-th block and the k-th replication,
$\bar{X}_{...}$ = overall average for the covariate X, and
ε_{ijk} = random error of the plot associated with each Y_{ijk} observation.

Subsequently, for the characteristics in which the effect of IBW was not detected ($P > 0.05$), variance analysis (ANOVA) was performed to verify the effects of the treatments (adopting the statistical model above) without the use of a covariate.

Because post-hoc power-of-the-test was less than 70% for Enterobacteriaceae counts and HV:DC ratio, a 10% level of type I error was used as the critical value in assessing these variables. Comparisons of treatment means for the other variables were performed using *t*-tests with a 5% level of probability. Statistical analyses were performed using SAS university edition (SAS Inst. Inc., Cary, North Carolina, USA).

Results and Discussion

There was no effect of treatments on DM, MM, and concentrations of Ca^{2+} and P of the metatarsal bone of the piglets. However, there was an effect on fresh weight, dry weight and length of this bone (Table 2).

Table 2 Means of physicochemical composition characteristics, fresh and dry weight, and metatarsal length of piglets as affected by the sources of calcium and phosphorus

Items	Dietary treatments				SE	*P*-value
	D1	D2	D3	D4		
Dry matter, %	76.28	74.37	73.38	71.01	1.708	0.745
Mineral matter, %	46.51	50.89	52.10	48.29	0.898	0.143
Ca^{2+} concentration, g/kg	164.22	167.23	169.51	157.86	1.959	0.200
P concentration, g/kg	172.01	172.24	172.04	172.04	0.448	0.997
Fresh weight, g*	5.84[a]	5.80[a]	3.97[b]	5.36[a]	0.194	0.012
Dry weight, g*	4.71[a]	4.68[a]	3.16[b]	4.09[a]	0.135	0.002
Length, mm*	54.73[a]	52.70[a]	45.24[b]	50.81[a]	0.817	0.004

*Analysis of covariance with the regression on initial bodyweight homogeneous across treatments
Ca^{2+}: calcium, P: phosphorus, D1: calcitic limestone + monodicalcium phosphate , D2: calcitic limestone, calcitic seaweed + monodicalcium phosphate, D3: similar to D2 but with -30% less calcium, D4: calcitic seaweed + monodicalcium phosphate
[a,b] Within a row, values with a common superscript were not different with probability *P* =0.05

There was no difference among treatments on BMD. However, the treatments affected the area, BMC, Seedor index and maximum force applied (MFA). D3 had lower area and Seedor index compared with the other treatments (Table 3). D1 and D2 had greater BMC compared with D3. D4 had intermediate BMC between D2 and D3, with the BMC not different from either of these treatments. However, MFA was greater for D2 and D4 than for the D1 and D3 (Table 3).

Table 3 Means of densitometry, density and metatarsal resistance characteristics of piglets as affected by the sources of calcium and phosphorus

Items	Dietary treatments				SE	*P*-value
	D1	D2	D3	D4		
Area, cm^2**	3.30[a]	2.87[b]	2.47[c]	2.92[b]	0.059	0.001
BMC, g*	0.520[a]	0.463[ab]	0.392[c]	0.441[bc]	0.010	0.011
BMD, g/cm^2	0.148	0.161	0.156	0.150	0.002	0.242
Seedor index, mg/mm*	107.77[a]	109.53[a]	83.90[b]	106.46[a]	2.773	0.014
MFA, N**	401.68[b]	587.90[a]	370.16[b]	520.79[a]	13.117	<0.001
MFA, kg**	40.96[b]	59.95[a]	37.75[b]	53.11[a]	1.337	<0.001

*Analysis of covariance with the regression on initial bodyweight homogeneous across treatments
**Analysis of covariance with the regression on initial bodyweight heterogeneous across treatments, means adjusted to an initial body weight of 5.42 kg
BMC: bone mineral concentration, BMD: bone mineral density, MSF: maximum force applied, D1: calcitic limestone and monodicalcium phosphate , D2: calcitic limestone, calcitic seaweed and monodicalcium phosphate, D3: s D2 but with -30% less calcium, D4: calcitic seaweed and monodicalcium phosphate
[a,b,c] Within a row, values with a common superscript were not different with probability *P* =0.05

All the piglets remained healthy. The results showed that Ca^{2+} sources were similarly effective in maintaining the MM content and the concentrations of Ca^{2+} and P. Despite this, CS is an organic Ca^{2+} source with greater bioavailability compared with CL (Avelar *et al.*, 2009). This may explain why D3 piglets maintained their MM and Ca^{2+} concentrations. Moreover, bones are the largest reserve of Ca^{2+} in the body, which ensures the maintenance of Ca^{2+} and P homeostasis in addition to functioning in the structure and support of the body (Gerlinger *et al.*, 2019).

Possibly the minerals in CS were not used for other organic functions of piglet metabolism, resulting in greater concentration in bone tissue and reduction in other tissues (liver, heart, and kidney), altering MM (Avelar *et al.*, 2009). The current findings for MM were similar to those of González-Vega *et al.* (2016), who evaluated six diets with various calcium levels and reported values of the average ash content in the metacarpal (52.6%) and femur (56.75%), with concentrations of Ca^{2+} (37.68%) and P (17.90%) in the piglet femur.

The weight and length of the bone are related to the amount of mineral deposited, which may explain the results found in D3, showing that the reduced Ca^{2+} supply affected the its deposition in bone. Thus, unbalanced diets or those that have lower levels of minerals that are important for bone health result in impaired bone formation and reduced growth (Santana *et al.*, 2017), which promotes change in the size, shape, and cells of the bone matrix (Barcellos *et al.*, 2007).

The literature is limited in studies of CS and its effects on bone parameters of starter phase pigs. Saraiva *et al.* (2009) tested various levels of total P and Ca^{2+} (0.68%) and reported average values for the ash content (50.37%) and Ca^{2+} (163.4 g/kg) in the metatarsal analogous with the current findings. However, Santana *et al.* (2017) tested different sources of Ca^{2+} in piglet diets and observed slightly higher values than the present study in metatarsal length, and its DM content and fresh weight.

Changes in BMC promote measurable changes in BMD, although the BMD values were not significant among the treatments. This observation may be related to the proliferation of osteoblasts stimulated by the chemical factor released by osteocytes that function as mechanical sensors and thus respond to dietary insufficiency to promote bone increment (Andreoli *et al.*, 2001). According to Cadore *et al.* (2005), BMD is a dynamic process of bone formation and remodeling and its maintenance is important for the prevention of bone malformation. However, resorption of bone tissue causes tissue deterioration, which was not observed for BMD in the present study.

Measurement of structural integrity of bone involves the application of mechanical tests. Bone ash content is directly related to mineralization, but the relationship with structural integrity is generally lower. Crenshaw *et al.* (2011) reported the potential appearance of bone damage because of malnutrition and that results obtained from a sample of one bone could be used to characterize the entire skeleton. Bollen and Bai (2005) observed reduced skeletal measurements of femur and skull in male rats fed diets containing low Ca^{2+} levels and controlled intake of diet.

Low Ca^{2+} intake impaired SI, MFA and BMC. These changes can cause negative changes in bone resistance to breaking, as reported by Barcellos *et al.* (2007). According to Suttle (2010), the swine metatarsus is a fast-growing bone that can be used as an indicator of resistance to breaking to reflect differences in the formation and growth of bone from variations in the deposition of minerals such as Ca^{2+}.

Rufino *et al.* (2017) reduced the amount of P and concomitantly of Ca^{2+} in piglet diets and observed lower bone resistance in the metacarpal of pigs. The reason that D3 and D4 showed lower MFA and their bones had less resistance to breaking involved factors such as bone length, diameter, weight and plasma Ca^{2+} concentration (Santana *et al.,* 2017). Besides being affected by bioavailability, Ca^{2+} and P can interact in bone metabolism (Schlegel & Gutzwiller, 2017).

No effect of treatments was observed in DM, MM, Ca^{2+} concentration in the heart, in MM in the liver, or in DM, MM, Ca^{2+} and P concentrations in the kidneys of the piglets. However, treatments affected the P concentration in the heart, and DM and concentrations of Ca^{2+} and P in the liver (Table 4). The results indicated that D2 had greater P concentration in the heart than D3 and D4. The D3 treatment produced more DM in the liver than D1. The Ca^{2+} concentration in the liver was higher for D4 compared with the other diets. The P concentration in the liver was higher in D4 compared with D1 and D3.

There is no evidence that piglets fed diets that supplied less Ca^{2+} had greater liver DM compared with those that met the Ca^{2+} requirement (Table 4). Increased protein, fat and carbohydrates in the liver may decrease its moisture content (Tajik *et al.*, 2012). Diets that restricted the amount of Ca^{2+} supplied to rats promoted greater fat mass (Bollen & Bai, 2005). More detailed studies are needed to assess Ca^{2+} kinetics and effects of Ca^{2+} sources on the physical composition of piglet body tissues because of a possible response of MM content to different sources of P (Queiroz *et al.*, 2008).

Table 4 Means of dry matter, mineral matter, and calcium and phosphorus concentrations in organs of piglets as affected by the sources of calcium and phosphorus

	Experimental treatments				SE	*P*-value
Heart	D1	D2	D3	D4		
Dry matter, %**	20.60	21.51	20.30	21.29	0.180	0.108
Mineral matter, %	5.56	5.23	5.39	5.13	0.116	0.601
Ca^{2+} concentration, g/kg	2.03	2.03	1.98	2.07	0.028	0.732
P concentration, g/kg	40.97^{ab}	49.98^{a}	35.27^{b}	33.83^{b}	1.938	0.005
Liver						
Dry matter, %**	25.93^{b}	26.22^{ab}	27.24^{a}	26.80^{ab}	0.288	0.045
Mineral matter, %	5.84	5.66	6.53	6.67	0.290	0.535
Ca^{2+} concentration, g/kg	1.98^{b}	2.02^{b}	2.00^{b}	2.26^{a}	0.037	0.043
P concentration, g/kg	61.56^{b}	63.44^{ab}	61.02^{b}	71.10^{a}	1.706	0.001
Kidney						
Dry matter, %**	20.68	20.33	20.04	19.93	0.261	0.742
Mineral matter, %	7.90	7.56	8.39	8.32	0.151	0.209
Ca^{2+} concentration, g/kg	2.14	2.18	2.13	2.21	0.030	0.753
P concentration, g/kg	50.98	67.85	47.33	67.32	4.267	0.163

**Analysis of covariance with the regression on initial bodyweight heterogeneous across treatments, means adjusted to an initial body weight of 5.42 kg

Ca^{2+}: calcium, P: phosphorus, D1: calcitic limestone and monodicalcium phosphate , D2: calcitic limestone, calcitic seaweed and monodicalcium phosphate , D3: like D2 but with -30% less calcium from calcitic limestone and monodicalcium phosphate, D4: calcitic seaweed and monodicalcium phosphate

[a,b] Within a row, values with a common superscript were not different with probability *P* =0.05

Previously, MM in the internal organs of piglets has been related to the dietary source of Ca^{2+}, its bioavailability and the level at which it was provided (Santana *et al.*, 2017). The percentage of MM in organs is related to the use of the mineral by the tissue. Absorbed minerals may not have been fully used by the metabolism, which increases the concentration in the kidneys and liver, concomitantly in the MM content. Although the content of MM is influenced by age, the Ca^{2+} content remains relatively constant, regulated by physiological processes (Field, 2000).

The results suggested that Ca^{2+} concentration is established in organs and tissues because the physiological response to Ca^{2+} is regulated by several factors (Gerlinger *et al.*, 2019). Calcium is found mainly in the endoplasmic reticulum and mitochondria (Bygrave & Benedetti, 1996), bones (99%) and serum (1%) (Beto, 2015). However, the amount of Ca^{2+} is in a range of 1,200 g in bone tissue (99% or 29.94 mol), 7 g in teeth (0.6% or 174.66 mmol), 7 g in soft tissues (0.6% or 174.66 mmol), 0.7 g in extracellular fluid (0.06% or 17.47 mmol), and 0.35 g in plasma (0.03% or 8.73 mmol) (Nordin, 1997). The present findings corroborate those reported by Moreira *et al.* (2004), who evaluated Ca^{2+} and P concentrations in tissues and obtained higher values for bone (83.02 and 75.35 mg/kg DM, respectively) compared with the Ca^{2+} concentration in the heart (0.32 mg/kg DM), and P concentration in kidney (8.91 mg/kg DM). Georgievskii *et al.* (1982) reported a concentration of Ca^{2+} (per 100 g of tissue) within a range of 8 to 25 mg in the heart, 10 to 30 mg in the liver and 6 to 20 mg in the kidney.

The treatments in this study did not affect (*P* >0.05) the pH of the digestive tract contents, except in the cecum and colon. D1 showed a higher pH of the cecal contents compared with D4. D2 had colon contents with a higher pH than D4 (Table 5). There was no effect (*P* >0.05) of treatments on the lactic acid bacteria count of the digesta. However, D4 showed a higher *Enterobacteriaceae* count in the jejunum compared with D1 and D3. A similar difference between D1 and D4 was observed for the pH of the digesta in the ileum. D3 had a greater *Enterobacteriaceae* count in the cecum compared with D1 (Table 5).

Table 5 Means of pH of the digestive tract content and lactic acid bacteria and Enterobacteriaceae count of piglets as affected by the sources of calcium and phosphorus

Items	Dietary treatments				SE	*P*-value
	D1	D2	D3	D4		
pH of the digestive tract content						
Stomach	3.95	4.08	3.59	3.80	0.138	0.640
Jejunum	5.97	6.24	6.36	5.87	0.087	0.200
Ileum	6.08	6.07	5.81	5.76	0.126	0.717
Cecum	5.59^{a}	5.39^{ab}	5.47^{ab}	5.26^{b}	0.037	0.028
Colon**	5.89^{ab}	6.13^{a}	5.97^{ab}	5.77^{b}	0.058	0.011
Lactic acid bacteria count, log_{10} CFU/g						
Jejunum	4.809	4.806	4.803	4.795	0.086	0.486
Ileum	4.817	4.816	4.822	4.794	0.067	0.250
Cecum*	4.784	4.776	4.804	4.791	0.090	0.252
Colon	4.802	4.801	4.799	4.815	0.067	0.424
Enterobacteriaceae count, log10 CFU/g						
Jejunum*	4.797^{c}	4.813^{ab}	4.796^{bc}	4.824^{a}	0.059	0.020
Ileum*	4.798^{b}	4.809^{ab}	4.809^{ab}	4.820^{a}	0.078	0.071
Cecum	4.793^{a}	4.785^{ab}	4.800^{a}	4.763^{b}	0.096	0.067
Colon	4.809	4.795	4.796	4.789	0.094	0.475

*Analysis of covariance with the regression on initial bodyweight homogeneous across treatments
**Analysis of covariance with the regression on initial bodyweight heterogeneous across treatments, means adjusted to an initial body weight of 5.42 kg
D1: calcitic limestone and monodicalcium phosphate in diet, D2: calcitic limestone, calcitic seaweed and monodicalcium phosphate in diet, D3: similar to D2, but with -30% less calcium, D4: calcitic seaweed and monodicalcium phosphate in diet
[a,b] Within a row, values with a common superscript were not different with probability *P* =0.05

The results suggested a minor role for the pH of the digestive tract in the modulation of the LAB microbiota. Because pigs fed diets containing CL + DP or CL + CS + DP showed greater pH of the digestive tract content may be a result of the Ca^{2+} and P absorption. The results were inconsistent with the study by González-Vega *et al.* (2014), who reported higher pH values in piglets fed diets containing CS than in piglets fed Ca^{2+} carbonate. These authors thought this was because Ca^{2+} and P absorption decreased from CS. However, Brun *et al.* (2014) stated that the Ca^{2+} absorption was reduced at low pH.

The pH values in the current study are in agreement with Rufino *et al.* (2017), who found no difference between treatments and intestinal segments for diets that met the supply of Ca^{2+} and P and those with reduction of these minerals. For Almeida *et al.* (2012), in a study with rats, *L. calcareum* (dose of 480 mg/kg) demonstrated a protective effect on low-intensity gastric lesions and increased the gastric pH. However, the pH of the digestive tract contents might vary depending on the diet/feed intake (extrinsic) and animal physiology (intrinsic).

Reduced pH inhibits the growth of pathogenic bacteria such as *Escherichia coli* and *Salmonella* sp., which are then unable to harm the gastrointestinal tract (Tran *et al.*, 2016). However, the authors did not find a plausible answer to justify the increase in *Enterobacteriaceae* in piglets receiving CS + DP owing to lower pH in the jejunum (5.87), cecum (5.26), and colon (5.77), where a higher pH (7.2 to 7.8) is speculated to provide an ideal environment for colonization of *E. coli* in intestinal villi (Gonzales *et al.,* 2013). Various studies have demonstrated changes in the microbial community with Ca^{2+} supplementation (Blavi *et al.*, 2018). However, the Enterobacteriaceae growth can be verified over a wide pH range (4.5 - 9.0) (Gonzales *et al.*, 2013).

The reduced pH of the digestive tract content may provide an appropriate environment for the development of beneficial bacteria and inhibit growth of pathogenic bacteria. The authors were not able to identify alterations in the intestinal microbiota for LAB. Despite the absence of significant differences between the treatments, except for the Enterobacteriaceae in the jejunum, reports in the literature indicate

that the amount of LAB was significantly higher than the pathogenic bacteria. However, one of the direct effects of Ca^{2+} supplementation was on the LAB that inhabit the hindgut (Blavi *et al.*, 2018). This was related to Ca^{2+} levels, sources, and the site of the gastrointestinal tract (Mann *et al.*, 2014).

However, the composition of the intestinal microbiota may present variations along the intestine because the population, quantity and microbial distribution are constantly changing their natural balance due to minimal variations in the physiological condition of the animal such as pH, bile and enzymatic secretion and microbial interactions (Oetting *et al.,* 2006).

There was no effect (P >0.05) of treatments on VH. However, D2 and DI produced greater CD in the duodenum (P =0.004) and ileum (P =0.011) compared with those that consumed D3 and D4 (Table 6). Feeding D3 caused a higher (P =0.091) VH:CD ratio in the duodenum compared with D2 (Table 6).

Table 6 Lsmeans of intestinal morphometry description of piglets according to experimental treatments[1]

Items	Dietary treatments				SE	*P*-value
	D1	D2	D3	D4		
Villus height, µm						
Duodenum	253.43	265.97	250.81	225.06	6.434	0.158
Jejunum*	275.62	254.8	259.04	227.20	10.710	0.484
Ileum	303.71	271.39	251.39	236.51	11.210	0.167
Crypt depth, µm						
Duodenum	97.89^{ab}	110.17^{a}	92.62^{b}	86.51^{b}	2.535	0.004
Jejunum	105.83	97.82	90.93	89.19	3.153	0.245
Ileum	117.95^{a}	101.88^{ab}	85.62^{b}	83.79^{b}	4.369	0.011
VH:CD ratio						
Duodenum	2.58^{ab}	2.43^{b}	2.70^{a}	2.59^{ab}	0.035	0.091
Jejunum*	2.61	2.60	2.82	2.53	0.049	0.505
Ileum	2.56	2.69	2.92	2.87	0.061	0.147

*Analysis of covariance with the regression on initial bodyweight homogeneous across treatments
D1: calcitic limestone and monodicalcium phosphate in diet, D2: calcitic limestone, calcitic seaweed and monodicalcium phosphate in diet, D3: similar to D2 but with -30% less calcium, D4: calcitic seaweed and monodicalcium phosphate in diet
[a,b] Within a row, values with a common superscript were not different with probability P =0.05

Piglets fed D4 had slightly smaller villi. This was supported by the greater Enterobacteriaceae count, especially enterotoxigenic *E. coli*, in which fimbriae F5, F6, and F41 colonize mainly in the distal jejunum and ileum, whereas F4 can colonize the entire jejunum and ileum (Sun & Kim, 2017), compromising intestinal architecture because the size, number, integrity and protection of the villi, in association, determine the intestinal architecture (Maiorka *et al.*, 2002).

Reduced villus size has been associated with post-weaning growth retardation in piglets (Anjos *et al.*, 2019). As a consequence, intestinal architecture had fewer secretory cells and reduced absorption capacity in the small intestine, and a larger amount of unabsorbed dietary material, which could act as a substrate for pathogenic bacteria and increase their growth (Gao *et al.*, 2013).

The variation in villi growth may be a result of greater cell proliferation, which is related to the higher CD to ensure an adequate epithelial renewal rate (Kisielinski *et al.*, 2002), which explains the results for D1 and D2. However, villus growth occurs when the mitosis rate is greater than that of extrusion or when extrusion is not occurring (Maiorka *et al.*, 2002). On the other hand, the capacity to absorb nutrients with lower energy losses owing to cell turnover is improved by greater VH and lower CD, that is, higher VH:CD ratio.

The VH:CD ratio values indicated a greater presence of mature and functional enterocytes (Tucci *et al.,* 2011). Because D3 showed higher VH:CD compared with those D2 suggested the need for further investigation on interactions between organic and inorganic sources, and calcium reduction in diets for

piglets. Supposedly, because D3 had a higher VH:CD ratio in an attempt to assimilate the reduced Ca^{2+} concentrations of unknown physiological mechanisms, Ca^{2+} metabolism or Ca^{2+} source.

To determine whether CS affected ROW, digestive and non-digestive organs were evaluated. However, there was no effect (P >0.05) of treatments on ROW (bodyweight percentage), but D3 showed greater (P =0.007) heart weight compared with those that consumed CL + DP (Table 7).

Table 7 Means of piglet relative organ weight according to experimental treatments

Items, % bodyweight	Experimental treatments				SE	*P*-value
	D1	D2	D3	D4		
Empty stomach	1.116	1.203	1.332	1.509	0.091	0.442
Empty SI + pancreas	5.283	5.269	5.725	5.672	0.180	0.688
Empty cecum	0.908	0.848	0.753	0.978	0.048	0.188
Empty colon*	3.248	3.246	3.687	3.356	0.133	0.474
Liver + gallbladder	2.581	2.739	3.018	2.931	0.122	0.642
Spleen	0.184	0.191	0.229	0.209	0.012	0.559
Kidneys	0.488	0.536	0.568	0.584	0.027	0.767
Heart**	0.492^{b}	0.509^{ab}	0.613^{a}	0.561^{ab}	0.019	0.007

*Analysis of covariance with the regression on initial bodyweight homogeneous across treatments
**Analysis of covariance with the regression on initial bodyweight heterogeneous across treatments, means adjusted to an initial bodyweight of 5.42 kg
D1: calcitic limestone and monodicalcium phosphate in diet, D2: calcitic limestone, calcitic seaweed and monodicalcium phosphate in diet, D3: similar to D2, but with -30% less calcium, D4: calcitic seaweed and monodicalcium phosphate in diet, SI: small intestine
[a,b] Within a row, values with a common superscript were not different with probability P =0.05

There seem to be no reports in the literature about relative organ weights from piglets fed different sources of calcium and *L. calcareum*. In general, piglets maintained similar organ weights, which demonstrated an apparently normal state of development. The weight of internal organs is associated with the growth, health and general condition of animal metabolism. In Knight and Dilger (2018), the effect of iron in diets for pigs was evaluated and a compensatory growth mechanism was observed after a period of feed restriction. This observation agreed with the present findings, because piglets that received D3 showed greater heart weight. In addition, different sources of P in pig diets affected the relative weight of the kidneys compared with a diet without inorganic P (Teixeira *et al.*, 2013).

The role of Ca^{2+} and P in animal metabolism and gene expression, involving absorption, resorption and bone remodeling influences the growth of body tissues (González-Vega *et al.*, 2016). The weight of the small intestine has been associated with feed intake and with development of intestinal villi. However, it was reported that Ca^{2+} propionate promoted the development of internal organs and the gastrointestinal tract (Zhang *et al.*, 2017).

Conclusions

These inconsistent findings suggest a need for further studies to better understand the interplay of effects of Ca^{2+} source and level on its metabolism.

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Authors' contributions

All the authors contributed equally and commented on the early and final version of the manuscript.

Conflict of Interest Declaration

There are no conflicts of interest.

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