



Impact of dietary *Laminaria digitata* with alginate lyase or carbohydrase mixture on nutrient digestibility and gut health of weaned piglets



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ARTICLE INFO

Article history:

Received 4 May 2023

Revised 4 May 2024

Accepted 7 May 2024

Available online 17 May 2024

Keywords:

Enzymes

Laminarin

Microbiome

Pigs

Seaweeds

ABSTRACT

Laminaria digitata is a brown seaweed rich in prebiotic polysaccharides, mainly laminarin, but its alginate-rich cell wall could compromise nutrient access. Carbohydrase supplementation, such as individual alginate lyase and carbohydrases mixture (Rovabio® Excel AP), could enhance nutrient digestibility and prebiotic potential. This study aimed to evaluate the effect of these enzymes on nutrient digestibility and gut health of weaned piglets fed with 10% *L. digitata*. Diets did not affect growth performance ($P > 0.05$). The majority of the feed fractions had similar digestibility across all diets, but the supplementation of alginate lyase increased hemicellulose digestibility by 3.3% compared to the control group ($P = 0.047$). Additionally, we observed that algal zinc was more readily available compared to the control group, even without enzymatic supplementation ($P < 0.001$). However, the increased digestibility of some minerals, such as potassium, raises concerns about potential mineral imbalance. Seaweed groups had a higher abundance of beneficial bacteria in colon contents, such as *Prevotella*, *Oscillospira* and *Catenisphaera*. Furthermore, the addition of alginate lyase led to a lower pH in the colon ($P < 0.001$) and caecum ($P < 0.001$) of piglets, which is possibly a result of released fermentable laminarin, and is consistent with the higher proportion of butyric acid found in these intestinal compartments. *L. digitata* is a putative supplement to enhance piglet gut health due to its prebiotic polysaccharides. Alginate lyase supplementation further improves nutrient digestibility and prebiotic potential. These results suggest the potential use of *L. digitata* and these enzymatic supplements in commercial piglet-feeding practices.

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Implications

Weaned piglets endure a critical stage in commercial pig production, due to their immature digestive and immune systems. *Laminaria digitata* can contribute to improved gut health of weaned piglets, avoiding the use of dietary zinc oxide, and its negative repercussions on the environment and public health. Enzymatic supplementation is a means to improve this nutritional potential,

releasing intracellular nutrients during digestion. We found that dietary seaweed improves the gut microbiome of piglets by increasing the abundance of fibre-fermenting microbial populations. Alginate lyase supplementation improves the digestibility of the cell wall fraction compared to the non-supplemented diet, demonstrating a beneficial effect.

Introduction

In recent times, there has been an increased trend towards local, high-quality feedstuffs for animal feeding due to climate change, political instability, the pandemic, and rising feedstuff

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prices (Komarek et al., 2021). Alternative feedstuffs such as insects or food industry by-products can contribute to sustainable production chains and circular economies (Muscat et al., 2021). In addition, modern feed formulation requires attention to both their nutritional composition and bioactive properties (Patience, 2018). This is particularly crucial in swine production, as the use of antibiotics and dietary zinc oxide as growth promoters has been restricted in many countries for human health and environmental reasons (Lopez-Galvez et al., 2021). Therefore, there is a need to explore alternative feed ingredients, such as microalgae (Martins et al., 2021a) and macroalgae (seaweeds) (Ribeiro et al., 2021), that possess both a favourable nutritional composition and health-promoting bioactive properties.

Seaweeds are multicellular organisms that can be categorised into three main groups, based on their pigmentation: red (*Rhodophyceae*), green (*Chlorophyceae*) and brown (*Phaeophyceae*) (Costa et al., 2021a). The worldwide cultivation of *Laminaria/Saccharina*, which are brown seaweeds, in 2019 was 12 273 748 tonnes, originating mostly from China (89%), Korea (10%) and Japan (0.27%), with European countries having comparatively minor contributions (Cai et al., 2021). The *Ascophyllum*, *Laminaria* and *Macrocystis* genera have been used in animal nutrition due to their interesting nutritional and bioactive properties. While they generally have low crude fat and CP contents (up to 5% and 5–13% on a DM basis, respectively) compared to green or red seaweeds, their fat is rich in *n*-3 polyunsaturated fatty acids, including eicosapentaenoic acid (20:5n-3), and their polysaccharides, such as laminarin, exhibit prebiotic properties (Makkar et al., 2016). The gross energy (GE) content of *Laminaria* sp. (12.7 MJ/kg DM) is closer to that of sugarcane molasses (14.9 MJ/kg DM) than to maize (18.8 MJ/kg DM) (Sauvant et al., 2004; Corino et al., 2019). Brown seaweeds can also have phlorotannins that can influence the digestive processes in monogastrics and ruminants alike (Belanche et al., 2016; Ford et al., 2020). *Laminaria digitata* is a brown seaweed, native to the North Atlantic coast (e.g. Ireland, Iceland, Norway), with long and smooth laminated blades that have been studied for its prebiotic polysaccharides (laminarin and fucooidan) and their potential to improve weaned piglet gut health, as demonstrated by the work of O'Doherty et al. (2021). However, using intact *L. digitata* biomass as a feedstuff could provide an alternative to conventional feedstuffs while taking advantage of its prebiotic properties.

Seaweeds have many beneficial properties for animal nutrition, but their recalcitrant cell wall can have anti-nutritional effects for monogastric animals. For instance, Brugger et al. (2020) found that up to 5% inclusion of *Laminaria japonica* reduced crude fibre digestibility in weaned piglet diets. This is due to the high content of non-starch polysaccharides in seaweed, which present a challenge for piglet digestive capacity. Exogenous enzyme supplementation can help to solve this problem. An earlier review by Makkar et al. (2016) suggested that this could be a viable option. Our team recently demonstrated that a recombinant alginate lyase was able to partially degrade the cell wall of *L. digitata* *in vitro*, thus releasing intracellular nutrients (Costa et al., 2021b). This is especially relevant since laminarin, a β -glucan, is located inside the macroalgal cells (O'Doherty et al., 2021). Therefore, the objective of the current study was to evaluate the effect of including a high level of dietary *L. digitata* (10%) and carbohydrase supplementation on nutrient digestibility and gut health variables of weaned piglets. This level of incorporation is more than double of what could be considered a supplement (< 3%) and at a level that unequivocally provides enough prebiotic substances, in addition to substrates for the supplemented enzymes, allowing their evaluation. The mode of action of enzymatic supplementation, either targeted, using a preselected recombinant alginate lyase, or untargeted, using a commercial mixture of carbohydrases, was also considered.

Material and methods

Animal trial: piglets, diets and slaughter

Experimental procedures have been previously described in a companion paper (Ribeiro et al., 2022) and are briefly described here for contextual purposes. This experiment was performed in the Animal Production Department of the School of Agriculture (Instituto Superior de Agronomia) of the University of Lisbon, Portugal. All experimental procedures were approved by the Portuguese Veterinary Authority (Direção Geral de Alimentação e Veterinária), through process 0421/000/000/2020, following European Directive 2010/63/EU.

Forty entire male piglets (Large White \times Duroc crossbred) were bought from a commercial farm at 35 days of age and 10.49 ± 0.62 kg (mean \pm SD) live weight. They were randomly divided across four experimental groups (n = 10) fed different diets (Table 1): control (a standard maize-wheat-soybean meal diet), LA (10% *L. digitata* replacing control), LAR (LA + 0.005% Rovabio[®] Excel AP) and LAL (LA + 0.01% alginate lyase described by Costa et al. (2021b)). The enzymatic activities of the commercial enzyme mix Rovabio[®] are xylanase, β -glucanase, cellulase, pectinase, protease and others including endo-1,4 β -mannanase, β -mannosidase and α -galactosidase. Its inclusion level was based on the manufacturer's recommendations. The recombinant alginate lyase was picked from a short list of eight enzymes (cellulase, laminarinase A, endo-gulonate lyase, β -1,3-1,4-glucanase P2, alginate lyase, cellobiohydrolase, lytic transglycosylase A, α -L-fucosidase C) based on their ability to degrade the algal cell wall (measured by fluorescence intensity and release of reducing sugars) and release intracellular nutrients (such as fatty acids). Each piglet was individually housed in a metabolic cage with free access to water and was fed with the corresponding diet on a pair-feeding basis (50 g/kg live weight/day). After a 5-day adaptation period to diets and experimental conditions, the trial lasted for 2 weeks, divided into two periods (P1 and P2). Daily tasks included: visually classifying faecal consistency (0- normal, 1- soft, 2- diarrhoea, 3- severe diarrhoea), recording feed refusals, sampling faeces and urine. Piglets were weighed at the beginning and end of each week.

At the end of the trial, piglets were slaughtered by electrical stunning followed by exsanguination, according to commercial practices. The gastrointestinal tract was then sampled. Viscosity of the small intestine contents (duodenum plus jejunum, and ileum) was measured using a viscometer adjusted to 6 rpm and 23 °C, after 10 min of centrifugation at 18 144 g. Gastrointestinal contents pH was recorded using a glass electrode pH meter (Metrohm 744, Metrohm AG, Herisau, Switzerland). Caecum and colon contents were removed for gas-chromatography analysis of volatile fatty acids (VFAs) following a previously published method (Martins et al., 2022). Histological measurements were taken on the three segments of the small intestine (duodenum, jejunum and ileum), following standard procedures (Tonel et al., 2010).

Proximal analysis of feed, faeces and urine

Proximal analysis of feed and faeces followed previously published methods (EGRAN, 2001; Ribeiro et al., 2022). Faecal samples were dried at 60 °C for 72 h in an oven with ventilation. Diets (in triplicate) and dried faecal samples (in duplicate) were ground with a 1 mm diameter mesh in a cross-beater mill and analysed, for DM, ash, organic matter (OM), CP, crude fat and GE contents, following published guidelines (EGRAN, 2001). Urine nitrogen was also analysed using the Kjeldahl method (Lordelo et al., 2008). Diets and faeces were analysed for NDF and ADF, which

Table 1

Composition and nutritional properties of control (wheat and maize-based), LA (10% *Laminaria digitata*), LAR (LA + 0.005% Rovabio® Excel AP) and LAL (LA + 0.01% alginate lyase) diets for weaned piglets.

Item	<i>Laminaria digitata</i>	Control	LA	LAR	LAL
Ingredients (g/kg)					
Wheat	–	437	394	393.95	393.90
Corn	–	150	136	136	136
Soybean meal 44	–	250	226	226	226
Sweet whey powder	–	100	90	90	90
Sunflower oil	–	30	27	27	27
<i>Laminaria digitata</i>	–	–	100	100	100
L-Lysine	–	5	4.5	4.5	4.5
DL-Methionine	–	1	0.9	0.9	0.9
L-Threonine	–	1	0.9	0.9	0.9
Calcium carbonate	–	5	4.5	4.5	4.5
Dicalcium phosphate	–	13	11.7	11.7	11.7
Sodium chloride	–	3	–	–	–
Vitamin-mineral premix ¹	–	5	4.5	4.5	4.5
Rovabio® Excel AP	–	–	–	0.05	–
Alginate lyase	–	–	–	–	0.10
Chemical composition (g/100 g DM)					
DM (%)	90.8	89.4	89.6	89.7	89.5
Ash	17.4	5.9	6.4	6.5	6.3
Organic Matter	82.6	94.1	93.6	93.5	93.7
Crude Fat	1.3	3.9	4.0	4.6	4.1
Gross Energy (MJ/kg DM)	12.8	18.4	18.0	18.0	18.1
CP	4.8	18.5	17.0	17.0	17.4
NDF	–	18.4	21.1	21.0	21.1
ADF	–	3.5	2.7	2.7	2.7
Glucans (g/ 100 g DM)					
α-glucans	8.6	0.2	1.5	1.6	1.6
β-glucans	35.8	0.9	8.4	3.2	8.5
Total glucans	44.4	1.2	9.9	4.8	10.1
Mineral profile (mg/kg DM)					
Br	474	15.1	83.1	80.8	87.7
Ca	8 819	17 445	16 022	16 675	15 931
Cu	2.88	274	244	269	236
I	4 399	9.56	652	647	713
Fe	144	304	226	253	246
Mg	5 637	1 751	2 615	2 605	2 569
Mn	5.42	149	123	123	113
P	903	11 131	6 381	6 445	6 167
K	28 530	12 789	15 694	15 651	15 680
Na	22 627	4 542	6 647	6 462	6 321
S	7 653	3 094	4 787	4 726	4 550
Zn	28.1	229	254	269	233

¹ Vitamin-mineral premix, VitaTec®, provided by Tecadi, Santarém, Portugal. Per 1 kg of premix: Vitamin A – 3 000 000 IU, Vitamin D3 – 500 000 IU, Vitamin E – 10 000 mg, Vitamin B1 – 500 mg, Vitamin B2 – 1 000 mg, Vitamin B6 – 500 mg, Vitamin B12 – 5 mg, Vitamin H2 – 1 875 mg, Vitamin K3 – 500 mg, Vitamin B5 – 3 750 mg, Vitamin B3 – 6 250 mg, Vitamin B9 – 62.5 mg, Choline chloride – 50 000 mg, Cu – 38 750 mg, Zn – 27 500 mg, Mn – 12 500 mg, I – 200 mg, Se – 50 mg, Fe – 25 000 mg, butylhydroxytoluene – 50 mg.

were determined following the method described by Van Soest et al. (1991). Hemicellulose was calculated as NDF-ADF. The fatty acid, pigment and mineral profile of these diets, as well as the methodologies used, have been previously reported in a companion paper (Ribeiro et al., 2022). The glucan analyses (total glucans, α-glucans and β-glucans) were determined enzymatically using the reagents K-YBGL (Megazyme, Bray, Ireland), according to Garcia-Vaquero et al. (2021).

Microbiome analysis

Six piglets per group, with feed conversion ratios similar to the group mean, were selected for microbiome analysis similar to what has been previously described (Namted et al., 2022). This is the only analysis of the current study where the number of animals was reduced from the initial ten. Total bacterial DNA was extracted from the colon content samples using the HostZERO Microbial DNA kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. DNA quantity and quality were evaluated using a Nanodrop ND 1000 spectrophotometer (Nanodrop Tech-

nologies Inc., Wilmington, DE, USA). The DNA was amplified for the V3-V4 hypervariable regions of the 16S rRNA gene. Amplicons were produced using the primers Pro341F: 50-TCGTCGGCAGCGT CAGATGTGTATAAGAGACAGCCTACGGGNBGCASCAG-30 and Pro805R: 50GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACT ACNVGGGTATCTAATCC-30, using Platinum™ Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific, Monza, Italy). The libraries were prepared using the standard protocol for MiSeq Reagent Kit v3 and were sequenced on the MiSeq platform (Illumina Inc., San Diego, CA, USA).

Raw sequences are publicly available at NCBI Sequence Read Archive (SRA) under the accession number PRJNA954028.

Calculations and statistical analysis

All data, except the microbiome data, were analysed using the SAS software (v. 9.4; SAS Institute Inc., Cary, NC, USA). The calculations of total tract apparent digestibility (TTAD) coefficients were performed using the following equation: $((A-B)/A) \times 100$, where A is the ingested nutrient and B is the excreted nutrient. The coef-

ficients of nitrogen utilisation used for the nitrogen balance were: Nitrogen Retention Coefficient (**NRC**) = $((\text{Nintake} - \text{Nfaecal} - \text{Nurine}) / (\text{Nintake} - \text{Nfaecal})) \times 100$; and Practical Nitrogen Retention Coefficient (**PNRC**) = $((\text{Nintake} - \text{Nfaecal} - \text{Nurine}) / \text{Nintake}) \times 100$. Data were checked for normal distribution and variance homogeneity. The statistical analysis was performed using the PROC MIXED of the SAS software (Littell et al., 1998). The model considered the piglet as the experimental unit, and the diet and period (each trial week) as the main effects. Significantly different least square means were compared using the Tukey posthoc test. Significance was declared when $P < 0.05$.

Microbiota analysis was carried out using the DADA2 pipeline (Callahan et al., 2016), and taxonomic categories were assigned using the Silva Database (release 138.1) as a reference (Quast et al., 2013). Alpha (Shannon, Chao1 and Simpson indices) and Beta (calculated as Bray Curtis distance matrix) diversity, as well as the abundance of taxonomic categories, were analysed with R software 3.6, using the PhyloSeq (McMurdie and Holmes, 2013), Vegan (Dixon, 2003) and MicrobiomeMarker (Cao et al., 2022) packages.

Differences between groups regarding alpha diversity indices (Chao1, Shannon, and Simpson diversity) were tested using an ANOVA model including the diet and sequencing depth as a fixed factor. Posthoc Tukey test for significant differences among the diets were then tested. For beta diversity, a dissimilarity matrix was constructed using the Bray-Curtis distance matrix as a metric on centred log-ratio transformed values, and the results were plotted using a Non-metric multidimensional scaling plot. Differences were tested using a PERMANOVA (Adonis) model with 9 999 permutations, including diet as a factor. Differences between the diets were assessed using the pairwise Adonis test. For the differential analysis of taxa, the LEfSe algorithm implemented in the wrapper function of the microbiomeMarker (version 1.0.2) package was used at the genus level after the centred log-ratio transformation of the data. A Linear Discriminant Analysis (**LDA**)t represscore > 3 and P.adj < 0.05 were considered to identify the microbial biomarkers of each diet (Segata et al., 2011).

Additionally, the data obtained from the microbial characterisation of the 16S were used for the functional prediction of microbiome metabolism. This prediction was made using the Tax4Fun2 package (Ashauer et al., 2015). Functional profiles were predicted by aligning the 16S sequence of each taxon with the reference dataset to identify the nearest neighbours. Subsequently, the metabolic potential was calculated by associating the sequence abundance with KO databases (Kyoto Encyclopaedia of Genes and Genomes). The abundance of metabolic pathways was then normalised based on the number of copies of the 16S rRNA gene of each taxon. Finally, the effect of the diet on the microbial functional profile was analysed using the LEfSe algorithm (LDA score > 2 and P.adj < 0.05).

Table 2

Effect of diet on growth performance of piglets fed with control (wheat and maize-based), LA (10% *Laminaria digitata*), LAR (LA + 0.005% Rovabio® Excel AP) and LAL (LA + 0.01% alginate lyase) diets.

Item	Control	LA	LAR	LAL	SEM	P-value
Initial weight (kg)	11.6	11.6	11.5	11.8	0.12	0.873
Final weight (kg)	17.0	16.7	16.9	16.8	0.16	0.936
Average daily gain (g)	385	359	383	359	9.6	0.653
Average daily feed intake (g)	548	569	571	571	11.1	0.866
Feed conversion ratio	1.45	1.62	1.51	1.64	0.049	0.478
Faecal consistency score ¹	0.314 ^A	0.035 ^B	0.064 ^B	0.135 ^{AB}	0.0382	0.038

Different superscripts indicate significantly different means ($P < 0.05$).

¹ Faecal consistency: 0- normal, 1- soft, 2- diarrhoea, 3- severe diarrhoea.

Results

Growth performance

Growth performance variables were, in general, unaffected by dietary treatments, including average daily gain, average daily feed intake and feed conversion ratio (Table 2). The only statistically significant effect concerning growth performance was found for the faecal consistency score ($P = 0.038$), which was higher (i.e. softer faeces) with control than with LA and LAR.

Total tract apparent digestibility

Total tract apparent digestibility coefficients are depicted in Table 3. There was no statistically significant interaction between period and diet ($P > 0.05$). The effect of the period was statistically significant ($P < 0.05$) regarding DM, OM and CP. In all instances, digestibility increased over time. Crude fat digestibility was increased in LAR compared to the other diets ($P = 0.001$). Regarding the fibrous fraction of the feed, there was a numerical increase in NDF digestibility in seaweed groups compared to control, with the maximum digestibility being achieved in LAL ($P = 0.188$). This relationship occurred with statistical significance in the hemicellulose fraction, with LAL having 4% increased digestibility compared to the control ($P = 0.047$). Acid detergent fibre digestibility was decreased by more than 20% in LA and LAR compared to control ($P < 0.001$). Alginate lyase supplementation increased ADF digestibility by more than 1.5-fold compared to either LA or LAR, despite being significantly lower when compared to the control. The remaining feed fractions were unaffected by dietary treatments.

Regarding macrominerals, the digestibility of potassium and phosphorous was significantly increased in LAL, and decreased in seaweed diets compared to control, respectively, with both coefficients being unaffected by period. In turn, three microminerals were affected by diet ($P < 0.05$). Iron digestibility was lower in LA compared to all other groups ($P < 0.001$). Manganese digestibility was lowered by dietary seaweed, regardless of enzymatic supplementation, compared to control ($P < 0.001$). Finally, zinc digestibility was higher in LAR than in control or LAL, with LA animals having intermediate values ($P < 0.001$). The period did not significantly influence any micromineral digestibility ($P > 0.05$) but influenced magnesium ($P = 0.040$) and sodium ($P < 0.001$) digestive coefficients, which increased with time.

Nitrogen balance

Nitrogen balance indicators are presented in Table 4. There was no interaction between period and diet, similar to what has been mentioned for TTAD. Period had a statistically significant effect

Table 3

Effect of diet and experimental period (P1 and P2) on total apparent tract digestibility coefficients (%) of piglets fed with control (wheat and maize-based), LA (10% *Laminaria digitata*), LAR (LA + 0.005% Rovabio® Excel AP) and LAL (LA + 0.01% alginate lyase) diets.

Item	Control	LA	LAR	LAL	P1	P2	SEM	P-value	
								Diet	Period
DM	85.9	84.6	85.4	85.8	84.9	86.0	0.32	0.616	0.041
Ash	70.3	69.2	71.2	72.3	70.1	71.5	0.72	0.625	0.241
Organic Matter	86.9	85.6	86.4	86.7	85.8	87.0	0.30	0.608	0.028
Crude Fat	69.8 ^B	70.0 ^B	74.7 ^A	70.9 ^B	71.2	71.5	0.49	0.001	0.743
Gross Energy	84.6	83.7	84.4	84.7	83.8	85.0	0.35	0.807	0.058
CP	81.2	78.2	80.5	79.5	78.8	80.9	0.46	0.149	0.013
NDF	75.5	77.7	77.8	79.4	77.3	78.0	0.58	0.188	0.516
ADF	40.8 ^A	13.8 ^C	14.2 ^C	21.1 ^B	22.5	22.4	1.50	<0.001	0.950
Hemicellulose	84.7 ^B	87.1 ^{AB}	87.2 ^{AB}	88.0 ^A	86.5	87.0	0.39	0.047	0.558
Minerals									
Ca	85.4	84.6	86.0	85.2	84.5	86.1	0.48	0.826	0.092
Mg	43.8	48.1	51.5	50.7	46.9	50.2	0.97	0.054	0.040
K	79.5 ^B	82.1 ^{AB}	83.0 ^{AB}	85.4 ^A	82.1	82.8	0.55	0.047	0.097
P	80.6 ^A	66.9 ^B	67.7 ^B	67.5 ^B	71.0	70.3	0.87	<0.001	0.546
Na	75.9	75.8	74.4	80.4	75.0	78.3	0.69	0.066	<0.001
S	77.6	76.7	77.0	76.5	76.7	77.1	0.48	0.889	0.693
Cu	58.0	53.9	58.5	53.6	57.3	54.6	0.76	0.123	0.057
Fe	56.1 ^A	40.8 ^B	50.5 ^A	51.6 ^A	49.3	50.2	0.97	<0.001	0.431
Mn	58.3 ^A	49.9 ^B	51.1 ^B	48.1 ^B	52.6	51.1	0.79	<0.001	0.256
Zn	21.4 ^C	35.7 ^{AB}	43.9 ^A	30.7 ^{BC}	33.2	32.7	1.40	<0.001	0.695

Different superscripts indicate significantly different means ($P < 0.05$). P1 = first experimental period; P2 = second experimental period.

Table 4

Effect of diet and experimental period (P1 and P2) on nitrogen balance of piglets fed with control (wheat and maize-based), LA (10% *Laminaria digitata*), LAR (LA + 0.005% Rovabio® Excel AP) and LAL (LA + 0.01% alginate lyase) diets.

Item	Control	LA	LAR	LAL	P1	P2	SEM	P-value	
								Diet	Period
Nitrogen intake									
g/d	17.0	15.8	15.8	16.1	13.9	18.5	0.30	0.070	<0.001
g/d/kg LW	1.18	1.11	1.11	1.12	1.07	1.19	0.013	0.055	<0.001
Nitrogen retention									
g/d	11.8	10.9	11.2	11.3	9.4	13.2	0.11	0.294	<0.001
g/d/kg LW	0.818	0.763	0.781	0.784	0.724	0.849	0.0127	0.382	<0.001
Nitrogen retention coefficients									
NRC (%)	84.3	87.6	87.6	88.5	85.8	88.2	0.58	0.093	0.017
PNRC (%)	68.8	68.5	70.5	70.3	67.7	71.3	0.66	0.668	0.002

P1 = first experimental period; P2 = second experimental period; PNRC = Practical Nitrogen Retention Coefficient; NRC = Nitrogen Retention Coefficient.

on all variables measured, including NRC and PNRC. All of the corresponding means were higher in the second period than in the first. There was a tendency ($P < 0.10$) for a higher nitrogen intake in piglets receiving the control diet. On the contrary, NRC had a tendency ($P = 0.093$) for increased retention in seaweed diets, of up to 4.2% increase in LAL compared to control.

Gut contents viscosity and pH

Viscosity and pH of gut contents are presented in Table 5. There was a numerical increase in ileal content viscosity in all seaweed diets compared to control. However, only LAR had a significant increase when compared to control ($P = 0.032$). Regarding the

Table 5

Effect of diet on viscosity and pH of gastrointestinal contents from piglets fed with control (wheat and maize-based), LA (10% *Laminaria digitata*), LAR (LA + 0.005% Rovabio® Excel AP) and LAL (LA + 0.01% alginate lyase) diets.

Item	Control	LA	LAR	LAL	SEM	P-value
Content viscosity (cP)						
Duodenum + jejunum	4.50	4.27	4.30	4.69	0.069	0.113
Ileum	4.74 ^A	5.34 ^{AB}	5.63 ^B	4.92 ^{AB}	0.121	0.032
Content pH						
Stomach	4.75	5.08	4.69	4.56	0.108	0.384
Duodenum + jejunum	5.24	5.51	5.56	5.50	0.065	0.301
Ileum	6.36	6.60	6.46	6.35	0.049	0.268
Caecum	5.73 ^B	6.07 ^A	5.69 ^B	5.31 ^C	0.051	<0.001
Colon	6.08 ^A	6.03 ^A	5.99 ^A	5.62 ^B	0.046	<0.001

Different superscripts indicate significantly different means ($P < 0.05$).

pH, there was no statistically significant effect of diet on the upper tract. It was, however, significantly influenced by diet in the distal part of the gastrointestinal tract. Caecum pH was highest when *L. digitata* was included without carbohydrases supplementation and lowest when alginate lyase was included in the diet ($P < 0.001$). In the colon, the lowest pH was found in LAL piglets, with the remaining diets showing similar values ($P < 0.001$).

Small intestine morphology

The morphological measurements taken on the small intestine of piglets are presented in Table 6. There was no statistically significant effect of diet on the villus height and width of the small intestine. Regarding crypt depth, there was a significant decrease in the jejunum with LA and LAR compared to the control ($P = 0.001$), which consequently increased the villus:crypt ratio in LA compared to the remaining groups ($P = 0.006$).

Table 6

Effect of diet on histological measurements taken on the small intestine segments (duodenum, jejunum and ileum) of piglets fed with control (wheat and maize-based), LA (10% *Laminaria digitata*), LAR (LA + 0.005% Rovabio® Excel AP) and LAL (LA + 0.01% alginate lyase) diets.

Item	Control	LA	LAR	LAL	SEM	P-value
Villus height (µm)						
Duodenum	379	370	370	348	12.5	0.854
Jejunum	475	487	424	450	10.3	0.129
Ileum	305	311	272	322	9.1	0.253
Villus width (µm)						
Duodenum	178	177	184	179	3.3	0.936
Jejunum	143	128	134	143	3.6	0.390
Ileum	164	160	163	156	3.3	0.869
Crypt depth (µm)						
Duodenum	547	510	502	469	12.3	0.169
Jejunum	356 ^A	280 ^C	300 ^{BC}	330 ^{AB}	7.8	0.001
Ileum	285	286	287	244	7.9	0.145
Villus:crypt						
Duodenum	0.708	0.739	0.739	0.785	0.0335	0.888
Jejunum	1.36 ^B	1.77 ^A	1.43 ^B	1.38 ^B	0.049	0.006
Ileum	1.09	1.14	0.967	1.36	0.054	0.071

Different superscripts indicate significantly different means ($P < 0.05$).

Table 7

Effect of diet on the volatile fatty acid profile of caecum and colon contents (mmol/ 100 mL) of piglets fed with control (wheat and maize-based), LA (10% *Laminaria digitata*), LAR (LA + 0.005% Rovabio® Excel AP) and LAL (LA + 0.01% alginate lyase) diets.

Item	Control	LA	LAR	LAL	SEM	P-value
Caecum						
Total	13.9	13.4	14.6	14.4	0.48	0.820
C2:C3	1.88	1.94	1.93	1.62	0.075	0.409
C2:C4	3.23	3.89	3.37	2.26	0.233	0.083
C3:C4	1.78	2.02	1.69	1.47	0.108	0.350
C2:Total	0.495	0.519	0.501	0.434	0.0124	0.072
C3:Total	0.273	0.274	0.265	0.277	0.0063	0.923
C4:Total	0.168 ^{AB}	0.147 ^B	0.165 ^{AB}	0.208 ^A	0.0079	0.035
C5:Total	0.054	0.057	0.061	0.082	0.0054	0.265
iC5:Total	0.008 ^A	0.005 ^{AB}	0.008 ^A	0.002 ^B	0.0008	0.040
Colon						
Total	15.1	14.1	14.6	15.3	0.51	0.862
C2:C3	2.58	2.20	2.00	1.93	0.104	0.111
C2:C4	3.71 ^{AB}	4.38 ^A	3.69 ^{AB}	2.57 ^B	0.203	0.012
C3:C4	1.45 ^{AB}	2.04 ^A	1.92 ^{AB}	1.34 ^B	0.088	0.007
C2:Total	0.554 ^A	0.548 ^A	0.519 ^{AB}	0.468 ^B	0.0108	0.014
C3:Total	0.228	0.255	0.269	0.246	0.0062	0.127
C4:Total	0.162 ^{AB}	0.132 ^B	0.150 ^B	0.193 ^A	0.0065	0.004
C5:Total	0.044 ^B	0.057 ^B	0.058 ^B	0.088 ^A	0.0042	0.001
iC5:Total	0.012	0.008	0.007	0.010	0.0011	0.487

Different superscripts indicate significantly different means ($P < 0.05$). C2, C3, C4, C5 and iC5 are acetic, propionic, butyric, valeric and isovaleric acids, respectively.

Volatile fatty acid analysis

The VFAs analysis of the caecum and colon contents is presented in Table 7. For the caecum, there was no statistically significant effect of diet in any individual VFA (data not shown). There was also a tendency for the ratio of C2:C4 and C2:Total to be the lowest in LAL piglets ($P = 0.083$ and $P = 0.072$, respectively). Finally, there was a statistically significant increase of C4:Total in LAL when compared to LA, with the remaining groups having intermediate values ($P = 0.035$). The ratio of iC5:Total was also significantly lower in LAL when compared to either control or LAR ($P = 0.040$). Regarding the colon, the ratios of C2:C4 and C3:C4 were both higher in LA compared to LAL ($P = 0.012$ and $P = 0.007$, respectively). The proportion of C4 and C5 on total VFA was highest in LAL compared to the remaining diets ($P = 0.004$ and $P = 0.001$, respectively). Regarding C2, its proportion was lowest in LAL and highest in control and LA ($P = 0.014$).

Colon microbiome

A total of 774 839 reads were attributed to a total of 1 115 Amplicon Sequence Variants distributed among samples as shown in [Supplementary Table S1](#). A sample was excluded from the analysis because of the low number of reads obtained by the sequencing. The relative rarefaction curves illustrated the tendency to plateau for all samples, suggesting that the sequencing depth was sufficient to describe the variability within the microbial communities analysed among the samples ([Supplementary Figure S1](#)). The taxonomic assignment allowed obtaining 14 phyla, 59 families and 147 genera. The most abundant phyla were *Firmicutes* ($91.33 \pm 6.41\%$), *Bacteroidota* ($2.77 \pm 2.83\%$) and *Euryarchaeota* ($2.20 \pm 3.27\%$). The most abundant families were *Lactobacillaceae* ($20.14 \pm 18.89\%$), *Lachnospiraceae* ($15.54 \pm 10.54\%$) and *Erysipelotrichaceae* ($11.98 \pm 8.04\%$). The most represented genera were *Lactobacillus* ($20.97 \pm 19.36\%$), *Streptococcus* ($10.88 \pm 11.30\%$) and *Catenisphaera* ($5.70 \pm 6.10\%$).

The alpha diversity indices of the four groups and the significant differences among them are reported in [Fig. 1](#). The diet significantly affected the Chao index ($P = 0.005$), while no effect was observed for the Shannon and InvSimpson indices. The contrasts showed that the control diet had a significantly lower Chao index compared with the LA ($P = 0.036$) and LAR diets ($P = 0.006$). The beta diversity was significantly affected by the diet ($P = 0.003$; $R^2 = 0.23$) as a result of the Adonis procedure. The pairwise Adonis test showed that the control diet was different to the LA ($P = 0.002$), LAR ($P = 0.001$) and LAL ($P = 0.01$) diets, while no difference was

observed for the other comparisons. In fact, the Non-metric multi-dimensional scaling plot evidenced a clear separation of the cluster of samples due to the control diet compared with the other diets ([Fig. 2](#)).

To identify which taxa contribute to the differences among the diets, the LEfSe algorithm on the data aggregated at Family and Genus levels was applied ([Fig. 3A and B](#)). At the family level, piglets from the control group had a higher abundance of *Lactobacillaceae* (LDA score = 5.29; $P_{\text{adj}} = 0.007$). The LA diet piglets were characterised by a higher abundance of *Erysipelatoclostridiaceae* (LDA score = 4.70; $P_{\text{adj}} = 0.006$), *Prevotellaceae* (LDA score = 4.35; $P_{\text{adj}} = 0.002$), *Succinivibrionaceae* (LDA score = 3.49; $P_{\text{adj}} = 0.027$), *Eubacteriaceae* (LDA score = 3.30; $P_{\text{adj}} = 0.024$). The LAR piglets were characterised by a higher abundance of *Monoglobaceae* (LDA score = 3.08; $P_{\text{adj}} = 0.005$). Finally, the LAL diet was characterised by a higher abundance of *Erysipelotrichaceae* (LDA score = 4.91; $P_{\text{adj}} = 0.024$) and *Coriobacteriaceae* (LDA score = 3.23; $P_{\text{adj}} = 0.037$). Furthermore, although they are not bacteria, the Chloroplast family and a genus belonging to the Chloroplast family were selected as microbial markers for the LA diet in which they were more abundant (Chloroplast family: LDA score = 3.06; $P_{\text{adj}} = 0.006$; genus of Chloroplast family: LDA score = 3.00; $P_{\text{adj}} = 0.006$).

At the genus level, piglets from the control group had a higher abundance of *Lactobacillus* (LDA score = 5.31; $P_{\text{adj}} = 0.007$) and *Marvinbryantia* (LDA score = 3.53; $P_{\text{adj}} = 0.002$). LA diet piglets were characterised by a higher abundance of *Catenibacterium* (LDA score = 4.73; $P_{\text{adj}} = 0.006$), *Prevotella* (LDA score = 4.19; $P_{\text{adj}} = 0.006$),

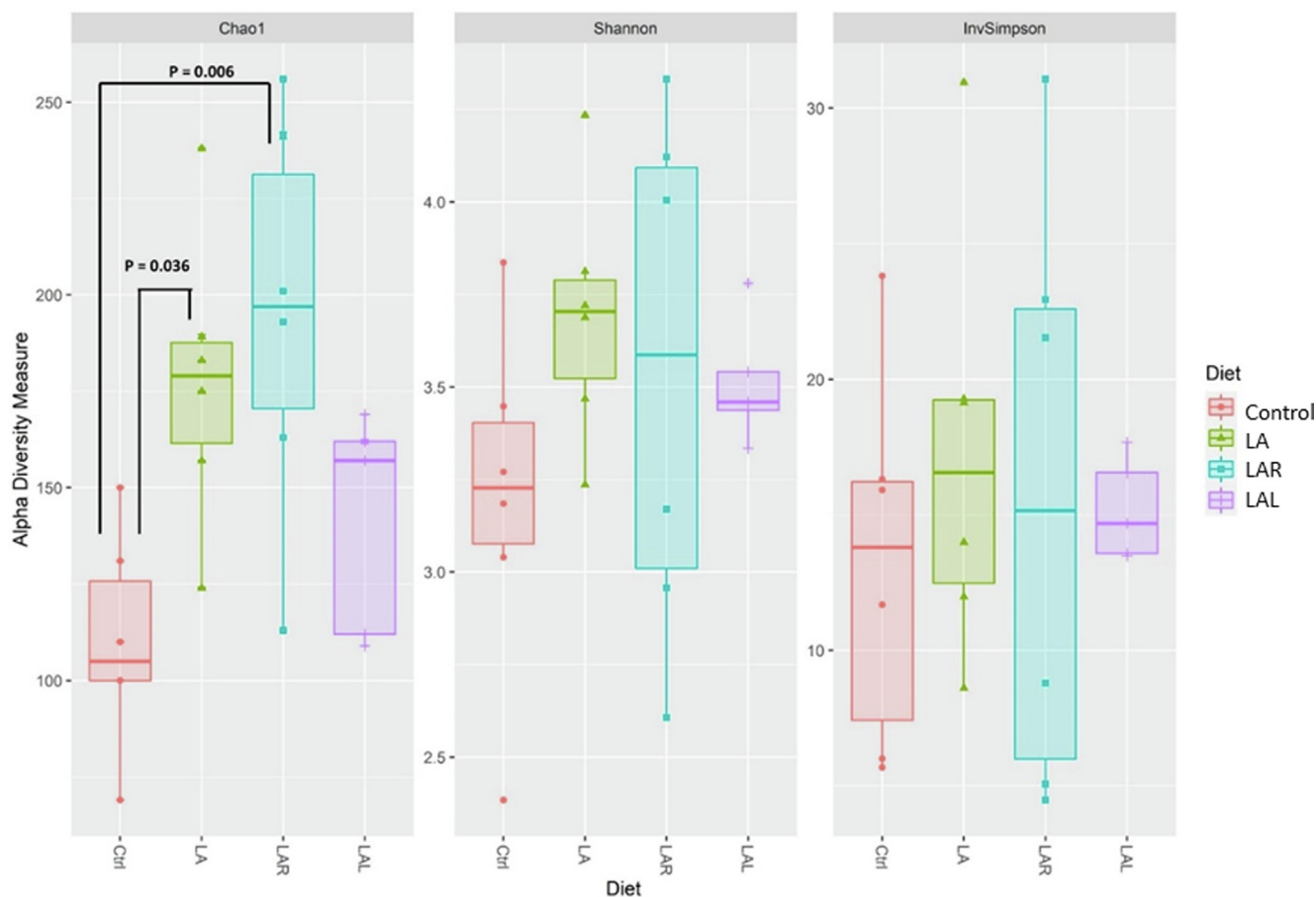


Fig. 1. The effect of the diets on the Chao1, Shannon and InvSimpson index values of colon contents from piglets fed with control (wheat and maize-based), LA (10% *Laminaria digitata*), LAR (LA + 0.005% Rovabio[®] Excel AP) and LAL (LA + 0.01% alginate lyase) diets.

adj = 0.02), *Succinivibrio* (LDA score = 3.45; P.adj = 0.03), *Intestinibacter* (LDA score = 3.32; P.adj = 0.04), *Pseudoramibacter* (LDA score = 3.25; P.adj = 0.03) and a genus belonging to the *Prevotellaceae* family (LDA score = 3.03; P.adj = 0.04). LAR diet piglets were characterised by a higher abundance of *Anaerostipes* (LDA score = 3.89; P.adj = 0.006), *Prevotellaceae_NK3B31_group* (LDA score = 3.87; P.adj = 0.003), *Oscillospira* (LDA score = 3.72; P.adj = 0.005), *Acetivomaculum* (LDA score = 3.67; P.adj = 0.007), *Erysipelotrichaceae_UCG-003* (LDA score = 3.28; P.adj = 0.013), *Oscillibacter* (LDA score = 3.28; P.adj = 0.008) and *Monoglobus* (LDA score = 3.03; P.adj = 0.005). The LAL diet piglets were charac-

terised by a higher abundance of *Catenisphaera* (LDA score = 4.80; P.adj = 0.015), *Syntrophococcus* (LDA score = 3.80 P.adj = 0.043), *Collinsella* (LDA score = 3.23; P.adj = 0.037) and *Erysipelotrichaceae_UCG-009* (LDA score = 3.08 P.adj = 0.016).

The functional prediction of the microbiome was carried out to identify potential different metabolic pathways. However, no difference was observed among the diets (Supplementary Figure S2).

Discussion

In the present study, we hypothesised that the combination of high levels (10%) of dietary *L. digitata* and carbohydrase supplementation would improve the nutrient digestibility of weaned piglet diets and gut health parameters. Due to the nature of the study, piglets were housed individually and fed in a controlled manner, which does not reflect commercial production conditions where piglets are housed in groups and fed *ad libitum*. Despite this limitation, our results showed that the inclusion of *L. digitata* had no detrimental effects on piglet growth performance, including average daily gain and feed conversion ratio. Moreover, dietary *L. digitata* significantly improved faecal consistency, although none of the groups had scores indicating diarrhoea. To our knowledge, brown seaweed biomass has seldom been used in inclusion levels similar to those of the present study in weaned piglet diets (Costa et al., 2021a). Previous research by Brugger et al. (2020) reported that feeding up to 5% *L. japonica* to weaned piglets improved the feed conversion ratio when fed *ad libitum*. However, further studies under commercial conditions are necessary to determine the effects of high levels of inclusion of whole *L. digitata* on piglet performance. Furthermore, it is important to point out that, due to the novelty of this seaweed, we approached its inclusion as a replacement for the basal diet. This implied reducing the inclusion of protein sources which consequently caused a reduction of the CP

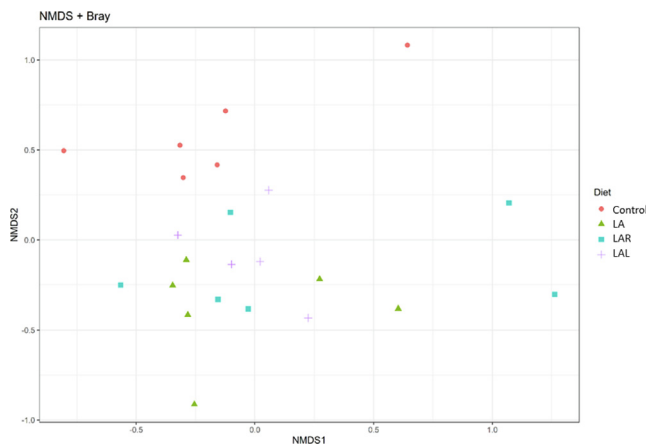


Fig. 2. Non-metric multidimensional scaling (NMSD) plot on Bray distance matrix on faecal samples of piglets fed with control (wheat and maize-based), LA (10% *Laminaria digitata*), LAR (LA + 0.005% Rovabio® Excel AP) and LAL (LA + 0.01% alginate lyase) diets.

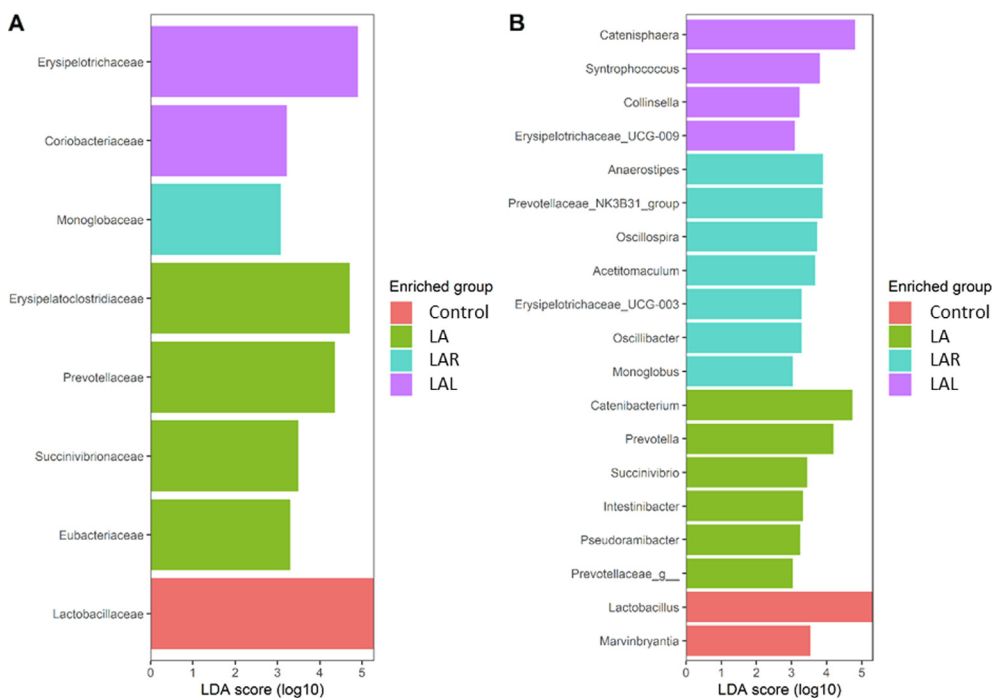


Fig. 3. Bar plot of Linear discriminant analysis (LDA) effect size (LEfSe) at Family (A) and Genus (B) levels for the faecal microbiota of piglets fed with control (wheat and maize-based), LA (10% *Laminaria digitata*), LAR (LA + 0.005% Rovabio® Excel AP) and LAL (LA + 0.01% alginate lyase) diets. Statistical analyses were performed using a linear discriminant analysis Effect Size (LEfSe) implemented in the wrapper function included in the microbiomeMarker 1.0.2 package, aggregating the data at family and genus levels. A linear discriminant analysis (LDA) score of 3 and adjusted *P*-value < 0.05 were used as a cutoff to detect the microbial marker. Horizontal bars represent the effect size for each taxon.

content of the diets. However, this was a necessary compromise to allow the calculation of the digestible CP and GE of the seaweed, giving a primary insight into its nutritional value. Nonetheless, and despite nitrogen intake being lower in *L. digitata*-fed piglets, nitrogen retention and the TTAD of CP were seemingly unaffected by this reduction. Finally, the overall increase in digestibility coefficients from periods one to two suggests good adaptation of the piglets to the experimental diets.

The digestibility of the fibrous fraction of the feed was influenced by dietary treatments, despite the fact that the digestibility of most nutrient fractions was not significantly affected. Specifically, the TTAD of NDF was numerically higher by up to 4% in seaweed diets compared to the control, although this difference was not statistically significant. This may be explained by the numerically higher digestibility of the hemicellulose fraction of the feed, which showed statistical significance in LAL when compared to control, possibly indicating a beneficial action of alginate lyase. In addition, ADF digestibility was more than halved in LA and LAR diets compared to the control, but the inclusion of recombinant enzyme significantly improved this reduction. Taken together, these findings suggest that the alginate lyase in weaned piglet diets enhances the degradation of algal cell walls, in line with the results of an *in vitro* assay (Costa et al., 2021b) and previous studies by the authors that reported the effective degradation of microalgal cell walls with recombinant enzymes (Martins et al., 2021b). This could contribute to the release of beneficial bioactive intracellular nutrients, such as laminarin. Furthermore, the β -glucan fraction of the LAR diet was found to be less than half that of the other seaweed diets, which confirms the activity of the supplemented enzymes. The major β -glucan in seaweed is laminarin (Garcia-Vaquero et al., 2021), and the Rovabio® commercial enzyme mixture contains laminarinase and endo-1,3(4) β -glucanase (Adisseo, 2023). The activities of these enzymes are likely responsible for the reduced β -glucans in the LAR diet. We can expect that this would also occur in the piglet digestive tract, where the Rovabio® mixture should degrade laminarin.

Studies investigating the digestibility of whole brown seaweed biomass in weaned piglet diets are limited, but some have been conducted. For instance, Brugger et al. (2020) reported that including *L. japonica* in piglet diets at levels of up to 5% resulted in a 10% decrease in crude fibre digestibility, despite increasing DM digestibility. The authors attributed this decrease to the high content of non-starch polysaccharides in the seaweed that are undigestible by monogastric animals. This is supported by *in vitro* digestibility results obtained by Ford et al. (2020), who reported that the effects of whole brown seaweeds on DM digestibility are influenced not only by phlorotannin content but also by their fibre composition. These findings support our argument that algal cell wall degradation assisted by alginate lyase supplied *in vivo* could enhance nutrient digestibility. Previous studies have reported mixed effects of *Laminaria* sp. extracts containing laminarin and fucoidan on pig feed digestibility, with some showing antagonistic effects and others negligible effects (Reilly et al., 2008; Gahan et al., 2009; Lynch et al., 2010). O'Doherty et al. (2010) demonstrated an increase in organic matter, nitrogen and gross energy digestibility attributed to a *Lactobacillus*-promoting effect that supports efficient digestibility of plant cell walls. However, these results were not observed in our study, possibly because we used whole seaweed biomass instead of purified bioactive polysaccharides. This resulted in lower dietary CP in LA, LAR, and LAL, which influenced nitrogen intake but did not affect CP digestibility. Using the whole seaweed may have prevented similar increases in DM digestibility. Nonetheless, we observed high hemicellulose digestibility in diets containing *L. digitata*, with statistically significant increments in LAL compared to controls. This seems to reflect the effectiveness of the recombinant enzyme in improving cell wall component

digestibility and the fermentability of some seaweed polysaccharides, similar to what has been reported by Lynch et al. (2010). This could have counterbalanced the reduction of ADF digestibility, causing no significant differences in DM digestibility. These findings are consistent with the lower pH of large intestinal contents of piglets fed *L. digitata* diets supplemented with alginate lyase, which seems to reflect the fermentation of components including laminarin released via algal cell disruption.

We aimed to improve the availability of intracellular nutrients for digestion by targeting one of the main cell wall polysaccharides, alginate, with alginate lyase. However, this approach may have negative consequences. For instance, alginate lyase supplementation significantly increased potassium digestibility compared to the control diet, resulting in a theoretically absorbed amount of 2.9 times the daily requirements for a growing piglet between 11 and 25 kg (NRC, 2012). Potassium is an essential electrolyte for cellular osmotic pressure and body pH regulation, which is crucial to maintain in weaned piglet diets (Lawlor et al., 2020). Increased salinity has been shown to compromise the homeostasis of porcine microbiota *in vitro*, reducing carbohydrate fermentation and increasing the proportion of enterobacteria, leading to protein fermentation characteristic of (osmotic) postweaning diarrhoea (Rink et al., 2011). Therefore, the increased potassium availability resulting from alginate lyase supplementation must be considered when feeding *L. digitata* to weaned piglets, as it may potentially counteract the prebiotic effects of the seaweed.

This study found that the inclusion of seaweed in piglet diets resulted in decreased phosphorus digestibility compared to the control, regardless of enzymatic supplementation. Phosphorus is an important mineral, along with calcium, for the development of skeletal tissue and muscle deposition (Lawlor et al., 2020). Therefore, attention should be paid to the dietary phosphorus contents when using seaweed in piglet diets as low digestibility can result in higher excretion and environmental contamination (Lautrou et al., 2021). However, the lower phosphorous content in the seaweed diets may have caused a decrease in its digestibility rather than lower digestive availability. In the seaweed groups, manganese digestibility coefficients were lower than control, but this did not seem to have major detrimental effects on the data evaluated, possibly due to the lower concentration in the seaweed diets. However, zinc digestibility coefficients were higher in the seaweed groups. The higher availability of zinc in the seaweed diets may influence the gut microbiome as observed in the present study and, in turn, it could have positively regulated the gut health of the weaned piglet. Indeed, zinc is present in several metabolic pathways, being a key structural and functional component for hundreds of enzymes (Lee, 2018). Iron digestibility was lower in the diet without enzymatic supplementation, while Rovabio® and alginate lyase reached the levels of control, reflecting the activity of the enzymes. The low digestibility of minerals in seaweed diets may result from both the cell wall integrity in the diet without enzymes and the formation of insoluble complexes (Corino et al., 2019). This could be a possible cause of low crude ash availability in earlier studies with *Laminaria* sp. It is important to note that the treatment of the seaweed biomass used in this study, which was micronised in addition to being milled, may have contributed to not finding other major detrimental effects of seaweed on mineral availability. Regardless, the intricacies of the mineral composition of seaweeds must be considered when using them for animal nutrition. Future studies could consider balancing the mineral composition of seaweed-containing diets in order to ascertain causality associations.

The impact of experimental diets on small intestine morphology was minimal, with only the jejunum showing statistically significant effects. In this segment, seaweed diets reduced crypt depth compared to controls, resulting in an increased villus: crypt ratio in

LA compared to control. Weaning can lead to undernutrition, disrupting intestinal morphology by causing an increase in crypt depth and a decrease in villus height (Heo et al., 2013). Reilly et al. (2008) reported that dietary *L. digitata* extract, containing laminarin, reduced villus height in the duodenum and jejunum of weaned piglets. In this study, this could be due to increased viscosity or the effect of seaweed phlorotannins. However, there was no increase in viscosity in the duodenum and jejunum intestinal contents in our study, with the only increase found in the ileum in LAR compared to the control, which could explain the absence of a reduction in villus heights. No detrimental effect was identified as a result of dietary tannins in the present study. Thus, the crypt depth reduction found with dietary *L. digitata* could indicate its potential to improve intestinal structure and function.

The effect of dietary treatments on the VFA profile of the large intestine content was mainly observed in the colon. This finding is consistent with the results reported by Lynch et al. (2010), who reported that laminarin fermentation leads to increased proportions of butyric acid in the distal part of the intestine. As endogenous enzymes are unable to digest laminarin, it reaches the colon and is fermented therein. Our study found that butyric acid fermentation was reduced in the LA group compared to the LAL group, which supports this premise. Butyric acid is a major energy source for colon cells and promotes the growth of beneficial bacteria, making it beneficial for weaned piglets. Reilly et al. (2008) have also suggested that *L. digitata* extract is useful for preventing postweaning diarrhoea due to its positive effects on the gut microenvironment, which our data appear to support.

We hypothesised that feeding weaned piglets with *L. digitata* could modify their gut microbiome profile, as reported in other studies showing the efficacy of algal polysaccharide extracts (O'Doherty et al., 2021). To maximise its prebiotic activities, we included a commercial carbohydrase mixture (including β -glucanases) and a recombinant alginate lyase to break down the cell walls of *L. digitata*. The commercial mixture can potentially degrade the storage polysaccharide laminarin, reducing its amounts reaching the hindgut and being fermented. Alginate lyase degrades the main cell wall polysaccharide, providing possible prebiotic oligosaccharides in the process (Venardou et al., 2023) and making intracellular nutrients more available, including laminarin. Other components, such as *n*-3 poly-unsaturated fatty acids, are not expected to have major effects on the distal gastrointestinal tract microbiome, as their digestion and absorption occur in the upper tract (Lauridsen, 2020). Our results showed that the control group had reduced Chao index only compared to either LA or LAR, indicating that alginate lyase reduced microbial diversity to levels similar to those of control animals. The release of laminarin may have contributed to this, as it has been shown to have antimicrobial activity (O'Doherty et al., 2021). On the contrary, the increase in the Chao index and no differences in the Shannon and InvSimpson indices in the LA and LAR compared with the control suggested an increase in the presence of rare taxa in these two latter groups (Chao and Chiu, 2016). In general, the increase in bacterial richness has been associated with a more stable and mature microbiota due to an increase in bacterial functional redundancy which, especially under stressful situations including weaning, represents a good gut health marker (Motta et al., 2019; Luise et al., 2021). However, this assumption is still unclear in pigs as lower richness has been associated with an increase in average daily gain (Lu et al., 2018). In the present study, the higher availability of minerals in the treated groups may also have influenced microbial growth. The precise causes require further research. These findings support the positive impact of *L. digitata* extract on the gut microenvironment, promoting a healthy microbiome composition in weaned piglets.

The populations of *Lactobacillaceae* family and *Lactobacillus* genus were found to be higher in control piglets, and these bacteria

have been positively associated with piglet gut health and performance improvement in various circumstances. For example, they are found to be enhanced when piglets are fed with prebiotic feed ingredients such as chicory root (Uerlings et al., 2019) or supplemented with algal polysaccharides such as laminarin and fucoidan (Rattigan et al., 2020). However, the effect of these algal polysaccharides is somewhat contradictory, with some studies reporting an enhancement of these populations (Vigors et al., 2020b), while others indicate no effect (de Vries and Smidt, 2020). Moreover, the simultaneous supplementation of laminarin and fucoidan can cancel each other's positive effects, which may not be synergistic. For instance, supplementing pigs with fucoidan has enhanced *Lactobacilli* populations, and this effect was lost when fucoidan was combined with laminarin (O'Doherty et al., 2021). In our study, the lower proportion of these populations in piglets fed with *L. digitata* could be attributed to the higher availability of algal zinc, which has been found to reduce them when fed as zinc oxide (de Vries and Smidt, 2020). We also observed that LA piglets had an increased abundance of *Prevotellaceae* family bacteria (e.g., *Prevotella*), which are known to enhance seaweed digestibility, as seen in the seaweed-foraging North Ronaldsay sheep (Williams et al., 2013). Previous research has shown that this family is increased in the gut microbiome of piglets fed diets supplemented with laminarin, and they were positively associated with beneficial VFA, including butyric acid (Vigors et al., 2020b). *Succinivibrio*, which are also a polysaccharide fermenting and butyric acid-producing bacteria like *Prevotella*, were also highly abundant in LA piglets. In our study, we found that the hindgut of piglets fed with *L. digitata* supplemented with alginate lyase had an increased butyric acid concentration, while the enrichment with these microorganisms was observed in the animals fed without enzymatic supplementation. These findings suggest that the presence of fucoidan, a cell wall polysaccharide (Bruhn et al., 2017), increased *Prevotella*, and when laminarin had increased availability due to cell wall disruption, this effect was lost. This is supported by the increased abundance of chloroplast genetic information found in piglets fed with *L. digitata* without enzymatic supplementation, demonstrating that cell wall disruption was achieved to a higher degree in its counterparts. Additionally, the significant improvement of ADF digestibility with LAL compared to either LA or LAR, and the particularly higher hemicellulose digestibility with LAL, corroborate these microbiota results. Future studies could also consider evaluating phlorotannin content to evaluate its impact on microbial communities. It is also important to note that chloroplast information is often removed from microbial analysis (Keto et al., 2021). In the present study, it was maintained as a putative marker of undigested plant material (Crespo-Piazuelo et al., 2018).

Feeding piglets with wheat bran, which is known to have a high hemicellulose content (Sauvant et al., 2004), was found to increase the concentration of faecal butyric acid (Molist et al., 2010), which is a signal of increased *Prevotella* communities in the gut (Lyu et al., 2020). However, supplementing high-wheat bran diets with xylanase was reported to reduce the populations of fibre-fermenting bacteria, such as *Lactobacillus* and *Lachnospiraceae* (Lyu et al., 2020). Carbohydrases may act during digestion, allowing some digestion of fermentable polysaccharides by the microbiota of the small intestine. Alternatively, other microorganisms may be fermenting *L. digitata* components in the hindgut of enzyme-supplemented piglets. Indeed, supplementing the *L. digitata* diet with alginate lyase promoted an increase in the abundance of *Catenisphaera*, a group of bacteria that has been associated with improved gut health and piglet performance following probiotic supplementation with *Bifidobacterium animalis* (Pang et al., 2022), as well as with feeding high-fibre diets to pigs (Yang et al., 2022). Furthermore, this group increased the abundance of *Syntrophococcus*, *Collinsella* and *Erysipelotrichaceae*_UCG_009, which

are highly present in the gut of piglets fed with the prebiotic inulin (Wu et al., 2020), feed-efficient pigs (Vigors et al., 2020a), and nursery piglets supplemented with xylo-oligosaccharides (González-Solé et al., 2022), respectively. *Collinsella* is a genus normally present in pig gut and is higher in pigs fed with probiotics (Kiros et al., 2019). In humans, this genus has been highlighted as one of the major utilisers of lactose and carbohydrates (Walker et al., 2011; Bag et al., 2017). These findings support our hypothesis.

The study found that adding Rovabio® to *L. digitata* diets had similar effects on the microbiome as LA, up to a certain point. For example, while LA increased the abundance of *Prevotellaceae_g* bacteria, Rovabio® supplementation increased the *Prevotellaceae_NK3B31_group*. This enzymatic supplementation also led to an increased abundance of genera such as *Anaerostipes* (known to produce volatile fatty acids in response to dietary plant polysaccharides (Yang et al., 2019)), *Oscillospira* (positively related to piglet performance (Miragoli et al., 2021)), and *Acetivomaculum* (beneficial for piglet gut health (Lin et al., 2022)). Overall, the results suggest that the gut health of piglets in this study was positively impacted by the enzymatic supplementation. Interestingly, the addition of seaweed to the diet increased bacterial diversity, but this effect was counteracted by antibacterial nutrients, likely laminarin, resulting from cell wall disruption caused by alginate lyase. Regardless, it is important not to overstate the importance of such results given that there were no significant differences in the Chao index of these groups (LA and LAR).

In conclusion, our study provides evidence that incorporating up to 10% *L. digitata* in weaned piglet diets can enhance gut health while maintaining growth performance. However, it is important to consider that these findings were obtained under specific experimental conditions, such as individual housing and controlled feeding. Despite this, feeding piglets with high levels of *L. digitata* did not significantly affect nutrient digestibility, except for fibre, which showed reduced ADF digestibility. However, this was improved with alginate lyase supplementation. Interestingly, the high digestibility of algal zinc may prove useful in managing postweaning diarrhoea. It is noteworthy that increased mineral availability may require dietary formulation adjustments to prevent mineral imbalances. Our results demonstrate that the microbiome of piglets fed *L. digitata* diets was seemingly healthy, with LA and LAR treatments showing increased bacterial diversity. Chloroplast abundance was higher in the LA group, possibly indicating enzymatic activity from both LAR and LAL treatments. Future research could focus on investigating the effects of these diets in commercial settings and examining tissue metabolism using Omics. It would also be interesting to explore possible physiological adaptations to increased mineral abundance in piglets.

Supplementary material

Supplementary material to this article can be found online at <https://doi.org/10.1016/j.animal.2024.101189>.

Ethics approval

All experimental procedures were approved by the Portuguese Veterinary Authority (Direção Geral de Alimentação e Veterinária), through process 0421/000/000/2020, following European Directive 2010/63/EU.

Data and model availability statement

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Microbiome data (raw sequences) are publicly available at NCBI Sequence Read Archive (SRA) under the accession number PRJNA954028 (<https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA954028>).

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

The authors declare that they have no competing interests.

Acknowledgements

The authors acknowledge Teresa Costa from Indukern, Lda. (Sintra, Portugal), for the kind donation of Rovabio® Excel AP.

Financial support statement

This research was funded by Fundação para a Ciência e a Tecnologia (FCT), Portugal, through PTDC/CAL-ZOO/30238/2017 grant, associated with a postdoc contract to M.C., and PhD fellowship to D.M.R. (SFRH/BD/143992/2019). CIISA (UIDB/00276/2020), AL4AnimalS (LA/P/0059/2020), TERRA (LA/P/0092/2020) and LEAF (UIDB/04129/2020) grants, also from FCT, are acknowledged. Armin Mirzapour-Kouhdasht works within the project AMBROSIA,

funded by the Department of Agriculture Food and the Marine (DAFM) under the umbrella of the European Joint Programming Initiative “A Healthy Diet for a Healthy Life” (JPI-HDHL) and of the ERA-NET Cofund ERA HDHL (GA No. 696295 of the EU Horizon 2020 Research and Innovation Programme).

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