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Effect of dietary inclusion of Spirulina on production performance, nutrient digestibility and meat quality traits in post-weaning piglets

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Abstract

The effect of Spirulina (*Arthrospira platensis*), individually or in combination with two commercial carbohydrases, in piglet diets was assessed on growth performance, nutrient digestibility and meat quality traits. Forty post-weaned male piglets from Large White × Landrace sows crossed with Pietrain boars with an initial live weight of 12.0 ± 0.89 kg were used. Piglets were assigned to one of four dietary treatments ($n = 10$): cereal and soya bean meal base diet (control), base diet with 10% Spirulina (SP), SP diet supplemented with 0.005% Rovabio® Excel AP (SP + R) and SP diet supplemented with 0.01% lysozyme (SP + L). Animals were slaughtered after a 4-week experimental period. Growth performance was negatively affected by the incorporation of Spirulina in the diets, with an average decrease of 9.1% on final weight, in comparison with control animals. Total tract apparent digestibility (TTAD) of crude protein was higher ($p < .05$) in the control group than in other groups. In addition, lysozyme increased TTAD of crude fat and acid detergent fibre, relative to the SP and control groups, respectively. In addition, the incorporation of Spirulina, individually and supplemented with enzymes, did not impair meat quality traits. Surprisingly, no protective effect against lipid oxidation was observed with the inclusion of Spirulina in pork after 7 days of storage. This study indicates that growth performance of post-weaning piglets was impaired by the incorporation of 10% Spirulina in the diets, which is mediated by an increase in digesta viscosity and a lower protein digestibility, as a consequence of the resistance of microalga proteins to the action of endogenous peptidases. In addition, it also indicates that lysozyme, in contrast to Rovabio® Excel

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AP, is efficient in the degradation of Spirulina cell wall in piglet's intestine. However, the digestion of proteins liberated by Spirulina cell wall disruption is still a challenge.

KEYWORDS
digestibility, lysozyme, meat, piglets, Spirulina

1 | INTRODUCTION

Cereal grains and soya bean meal are, respectively, the main energy and protein sources used in pig feeding (FAO, 2004). Such ingredients are primarily produced in North and South America and used throughout the world in animal production. The high economic and environmental costs associated with the production and transport of such feedstuffs over large distances and their direct competition with human consumption have important implications for the sustainability of feed and animal production (Manceron et al., 2014). Therefore, alternative sources of protein with balanced amino acid compositions are urgently needed, as are sources of *n*-3 long-chain polyunsaturated fatty acids (PUFA), vitamins, minerals, carotenoids and bioactive compounds of interest in animal feeding (Florou-Paneri et al., 2014).

Microalgae have been characterized by having high protein, carbohydrate and fat contents, in many cases comparable or even higher than conventional feedstuffs, such as soya bean meal (Lum et al., 2013). One of the most used microalgae in both food and feed production is Spirulina (*Arthrospira platensis*). Spirulina is a cyanobacterium, also known as a blue-green microalga, and therefore an autotrophic prokaryote. It has relatively low cost and a high nutritional value, in particular high protein (50% to 70% of dry weight) and interesting lipid (5% to 14% of dry weight) contents (Gutiérrez-Salmeán et al., 2015; Hoseini et al., 2013; Madeira et al., 2017). However, microalgae in general have recalcitrant cell wall carbohydrates, with very difficult digestion and used by monogastrics, such as pigs (Tibbetts, 2018). Although the microalgae cell wall structure and composition are complexes and poorly studied, it is known that they have rigid components embedded within a polymeric matrix, containing cellulose and an additional tri-laminar sheath containing algaenan, a compound that confers resistance to enzymatic degradation (Gerken et al., 2012; Popper & Tuohy, 2010). To circumvent this problem, Carbohydrate-Active enZymes (CAZymes) that lyse cell wall complex polysaccharides may be advantageous to improve nutrient utilization of microalgae (Sander & Murthy, 2009). Lysozyme is a CAZyme that cleaves the peptidoglycan of prokaryote cell walls, thus leading to a better exposure of proteins and pigments to the endogenous repertoire of digestive enzymes (Al-Zuhair et al., 2016; Oliver & Wells, 2015).

Piglet feeding, from weaning to two months of age, is one of the most challenging aspects in swine industry. In fact, it is well known that an adequate feeding of piglets is mandatory to overcome weaning stress and to efficiently reach slaughter weight. Furthermore, it is known that body composition at the end of post-weaning period influences growth performance during the growing–finishing phase, as well as body composition when pigs reach 100 kg live weight (Collins et al., 2017; Fix et al., 2010). Interestingly, in Portugal and Spain, there is a tradition of consumption of spit-roasted piglet, a delicacy product particularly valued by consumers (Correia et al., 2017). Examples of such *gourmet* products include *Leitão de Negrais* and *Leitão da Bairrada* in Portugal, and *Cochinillo Asado* and *Cochinillo Segoviano* in Spain. Spit-roasted piglet production has become a booming industry in the recent years, due to the product added value and its high demand, constituting an alternative to standard pork production that uses carcasses of animals typically slaughtered at 100 kg live weight. Interestingly, production and meat quality traits of such production systems have still to be characterized.

In line with the above-mentioned, the aim of our study was to assess the effect of incorporating 10% Spirulina in piglet diets, supplemented or not with two exogenous CAZymes (a commercial mixture of carbohydrate-degrading enzymes and lysozyme), on growth performance, nutrient digestibility and meat quality traits in piglets. It was hypothesized that Spirulina, supplemented or not with exogenous enzymes (Rovabio® or lysozyme), can be a viable source of nutrients, in particular of proteins, for feeding post-weaning piglets, with the main focus on their growth performance, digestibility and meat quality. Such information is of value to the scientific community and to the piglet and pig production industries.

2 | MATERIALS AND METHODS

2.1 | Experimental design: Animals and diets

Forty male post-weaned piglets, sons of Large White × Landrace sows crossed with Pietrain boars, weaned at 28 days of age and with an initial live weight of 12.0 ± 0.89 kg (mean ± *SD*), were obtained from a commercial farm in Central Portugal. After an adaptation period of 2 days, piglets were evenly distributed into four homogeneous groups of 10 piglets, as previously described (Correia et al., 2017). Briefly, they were randomly allocated into pens (1.9 × 1.1 m) equipped with one stainless steel nipple and one creep feeder. The floor was made of plastic slats. All groups had a similar average weight. Piglets had *ad libitum* access to feed and water. Each group received one of the four experimental diets: cereal and soya bean meal-based diet (control), control diet with 10% of Spirulina (SP), control diet with 10% of Spirulina supplemented with 0.005% of Rovabio® Excel AP (Adisseo; SP + R) and control diet with 10% Spirulina supplemented with 0.01% of lysozyme (Sigma 62971; Sigma-Aldrich Ltd.; SP + L).

Diets were balanced for crude protein and essential amino acids. Freeze-dried Spirulina (*A. platensis*) powder provided by Sopropeche

(Wimille, France) was used. The CAZymes used were chosen according to the study of Coelho et al. (2020), and the incorporation level followed producer recommendations. The ingredient composition of the diets is described in detail in Table 1, and their chemical composition is fully presented in Table 2. For further information on the analysis conducted on feeds, see details below.

2.2 | Animal performance and sampling

Throughout the experiment, supplied feed was recorded daily, whereas refusals and piglets were weighed weekly, just before feeding, in order to calculate average daily feed intake (ADFI), average daily weight gain (ADG) and feed conversion ratio (FCR). All animals were slaughtered, after an experimental period of 28 days, using electrical stunning followed by exsanguination, according to standard procedures used in commercial abattoirs. Gastrointestinal tract was removed, and the length of the small and large intestines was recorded. Samples of *longissimus lumborum* muscle were collected from the right side of the carcass, vacuum packed and stored at −20°C until further analysis to assess the meat quality.

TABLE 1 Ingredients and feed additives of the experimental diets (g/kg, as fed basis)

	Diets			
	Control	SP	SP + R	SP + L
Wheat	439	460	460	460
Corn	150	170	170	170
Soya bean meal 48	250	110	110	110
Whey powder	100	100	100	100
Soya bean oil	30	30	30	30
Spirulina	0	100	100	100
Rovabio® Excel AP	–	–	0.05	–
Lysozyme	–	–	–	0.1
L-Lysine	5	6	6	6
DL-Methionine	1	1	1	1
L-Threonine	1	–	–	–
Calcium carbonate	5	6	6	6
Dicalcium phosphate	13	12	12	12
Sodium chloride	3	2	2	2
Vitamin–mineral complex[1]	3	3	3	3

Note: Dietary treatments: SP—Spirulina diet; SP + R—Spirulina diet supplemented with Rovabio® Excel AP; SP + L—Spirulina diet supplemented with lysozyme.

[1]Premix provided per kg of complete diet: vitamin A, 6,500 UI; vitamin D_3, 1,500 UI; vitamin E, 15 mg; vitamin K_3, 1 mg; vitamin B_1, 1 mg; vitamin B_2, 3 mg; vitamin B_6, 2 mg; vitamin B_{12}, 0.02 mg; pantothenic acid, 10 mg; nicotinic acid, 15 mg; folic acid, 0.5 mg, biotin, 0.03 mg; betaine, 115 mg; vitamin C, 20 mg; Copper, 100 mg; iron, 100 mg; iodine, 0.5 mg; manganese 50 mg; selenium, 0.15 mg; zinc, 100 mg; butylated hydroxytoluene, 3 mg.

To measure the viscosity of small intestine contents, samples were collected from the duodenum plus jejunum and from the ileum, and centrifuged for 10 min at 18,144 g, and the viscosity of sample's supernatant was measured, in duplicate, using a viscometer adjusted to 6 rpm and 23°C (Model LVDVCP-II; Brookfield Engineering Laboratories).

2.3 | Faecal scores and total tract apparent digestibility

The faeces were observed daily, on each pen, to evaluate consistency, according to the following scale: 0 (normal), 1 (soft faeces) or 2 (diarrhoea).

In order to evaluate the digestibility of the four diets, an external marker (chromium oxide) was used, as described by Clawson et al. (1955). During the last two weeks of the experiment, the marker was added to the diets in a proportion of 0.5%. After one week of adaptation, the faeces of each pen were collected twice a day for five consecutive days. Faeces were stored at −20°C until further analysis. Chromium oxide in the diets and faeces was evaluated according to Bolin et al. (1952), for the estimation of total tract apparent digestibility (TTAD) of dry matter (DM), organic matter (OM), crude protein (CP), crude fat (CF), neutral detergent fibre (NDF), acid detergent fibre (ADF) and energy (E).

2.4 | Diets and faecal analysis

The faeces were dried at 60°C for 72 hr. Diets and dried faecal samples were ground in 1-mm-diameter mesh mill and analysed, in duplicate, for DM, ash, CP (automated Kjeldahl method) and CF contents, following the methods described by AOAC (2000). NDF and ADF were performed sequentially using crucibles system by Van Soest et al. (1991). Energy was calculated by complete combustion of diets and faeces in an adiabatic calorimeter (Parr 1261; Parr Instrument Company).

The determination of amino acids in diets and Spirulina were performed following the pre-approved protocol by the European Commission (2009), and the results are shown in Table 2. For cysteine and methionine, an oxidation with performic acid was performed before hydrolysis. The hydrolysis of the samples consisted of an attack with hydrochloric acid at 110°C for 23 hr. The hydrolysed amino acids were separated using ion-exchange chromatography and determined by photometric detection after reaction with ninhydrin. Tryptophan was hydrolysed using an alkaline solution of barium hydroxide at 110°C for 20 hr and measured by HPLC with fluorescent detection.

Fatty acid methyl esters (FAME) of the experimental diets were analysed by one-step extraction and transesterification, using heneicosaenoic acid (21:0) methyl ester as the internal standard (Sukhija & Palmquist, 1988).

TABLE 2 Chemical composition of Spirulina and experimental diets

		Diets			
	SP powder	Control	SP	SP + R	SP + L
Metabolizable energy (kcal/kg DM)[1]	—	3,738	3,809	3,798	3,818
Proximate composition (g/100 g, as fed basis)					
DM	93.8	89.8	90.0	90.0	90.0
CP	60.1	17.9	18.1	17.9	17.8
NDF	—	14.6	11.9	11.9	11.8
ADF	—	4.21	3.97	4.06	3.94
Ash	6.70	5.11	4.76	4.89	4.75
Crude fat	6.84	5.28	5.62	5.87	5.80
Amino Acid composition (g/100 g, as fed basis)					
Alanine	5.21	0.957	1.14	1.14	1.13
Arginine	4.67	1.38	1.26	1.26	1.22
Aspartate	6.50	2.13	1.88	1.86	1.81
Cysteine	0.577	0.374	0.327	0.324	0.326
Glutamate	9.39	4.41	4.02	4.01	3.89
Glycine	3.38	0.909	0.910	0.907	0.899
Histidine	1.03	0.548	0.458	0.456	0.439
Isoleucine	4.15	1.03	1.03	1.03	1.02
Leucine	6.00	1.70	1.69	1.69	1.66
Lysine	3.19	1.48	1.45	1.50	1.53
Methionine	1.59	0.410	0.484	0.457	0.447
Phenylalanine	2.95	1.06	0.963	0.956	0.930
Proline	2.43	1.41	1.29	1.30	1.26
Serine	3.46	1.14	1.06	1.06	1.03
Threonine	3.32	0.913	0.854	0.850	0.846
Tryptophan	1.13	0.295	0.293	0.290	0.294
Tyrosine	3.00	0.736	0.735	0.730	0.710
Valine	4.66	1.14	1.17	1.16	1.16
Fatty acid composition (% total fatty acids)					
12:0	0.00	0.123	0.122	0.146	0.140
14:0	1.01	0.396	0.479	0.520	0.531
16:0	37.6	13.5	17.8	18.3	19.3
16:1*c*9	13.5	0.140	0.817	0.875	0.836
18:0	1.00	3.17	3.19	3.32	3.62
18:1*c*9	1.47	24.1	21.1	20.5	20.1
18:1*c*11	0.21	1.30	1.23	1.28	1.27
18:2*n*-6	17.0	48.6	43.1	42.0	39.1
18:3*n*-3	0.00	4.55	4.17	4.31	4.22
20:0	0.160	0.364	0.325	0.324	0.323
20:1*c*11	0.00	0.298	0.572	0.559	0.820
22:0	0.00	0.365	0.365	0.365	0.421
Pigments (μg/g)					
Chlorophyll *a*[2]	1,197	2.70	108	112	132
Chlorophyll *b*[3]	45	4.97	14.6	13.0	17.5
Total chlorophylls[4]	1,242	7.67	122	125	149

(Continues)

TABLE 2 (Continued)

		Diets			
	SP powder	Control	SP	SP + R	SP + L
Total carotenoids[5]	697	2.41	11.6	12.6	13.0
Total chlorophylls and total carotenoids[6]	1,939	10.1	134	138	162
Diterpene profile (μg/g)					
β-Carotene	233	0.160	3.57	3.14	2.15
α-Tocopherol	24.6	7.41	12.0	12.3	12.9
β-Tocopherol	0.907	0.676	0.254	0.215	0.213
γ-Tocopherol	0.932	1.05	0.997	1.05	0.925
α-Tocotrienol	n.d.	1.09	0.504	0.923	0.994
Estimation of the mineral composition (%)					
Ca	0.12	0.727	0.704	0.704	0.704
P	1.30	0.624	0.662	0.662	0.662
Na	0.45	0.187	0.192	0.192	0.192

Note: Dietary treatments: SP—Spirulina diet; SP + R—Spirulina diet supplemented with Rovabio® Excel AP; SP + L, Spirulina diet supplemented with lysozyme.

—Difficulties in application of the standard procedure.

n.d.—not detected.

[1]Metabolizable energy (kcal/kg DM) = 4412–11,06 × Ash (g/kg DM) + 3,37 × Crude Fat (g/kg DM) − 5,18 × ADF (g/kg DM) (Noblet et al., 1989).

[2]Chlorophyll *a* = 11.24 × A662 nm − 2.04 × A645 nm.

[3]Chlorophyll *b* = 20.13 × A645 nm − 4.19 × A662 nm.

[4]Total chlorophylls (C*a* + *b*) = 7.05 × A662 nm + 18.09 × A645 nm.

[5]Total carotenoids (Cx + c) = (1,000 × A470 nm − 1.90 × Ca − 63.14 × Cb)/214.

[6]Total chlorophylls and carotenoids = (Ca + b) + (Cx + c).

The pigments of diets were measured according to Teimouri et al. (2013), with slight modifications. Briefly, the samples were extracted with acetone and stored under agitation during overnight. The solutions were centrifuged at 3,345 *g* during 5 min, and then, absorptions were measured by UV-Vis spectrophotometry (Ultrospec 3100 pro; Amersham BioSciences). The pigment content was calculated using the equations previously described by Hynstova et al. (2018).

The quantification of tocopherols and tocotrienols in the diets involved a direct saponification, a single *n*-hexane extraction and analysis of the extracted compounds by normal-phase HPLC using fluorescence detection, as described in our previous publication (Prates et al., 2006).

2.5 | Meat colour and pH measurements

Meat colour was measured on the cut surface of *longissimus lumborum* section, 24 hr *post-mortem*, using a colorimeter (Minolta CR-300; Konica Minolta). Two measurements per sample were recorded according to the CIE L^* (lightness), a^* (redness) and b^* (yellowness) system, after 1 hr of air exposure and as previously described by Madeira et al. (2013). The pH of *longissimus lumborum* muscle samples at 24 hr *post-mortem* was measured using a pH meter equipped with a penetrating electrode (HI8424; Hanna Instruments).

2.6 | Meat lipid oxidation

The extent of meat lipid oxidation was evaluated at days 0, 3 and 7 *post-mortem* (storage at 4°C), by measuring thiobarbituric acid reactive substances (TBARS), following the spectrophotometric method described by Grau et al. (2000). TBARS values were calculated, in duplicate, from a standard curve constructed with 1,1,3,3-tetraethoxypropane, as a precursor of malonaldehyde, and the results were expressed as mg of malonaldehyde *per* kg of meat (Madeira et al., 2014).

2.7 | Cooking loss and shear force determinations

Frozen meat samples were thawed at 4°C overnight, weighed and cooked in a water bath at 80°C until reaching an internal temperature of 78°C, using a thermocouple (Lufft C120; Lufft) adjusted with a blank test. After two hours of cooling at room temperature,

samples were weighed and longitudinally cut in the fibre axis into 8–10 cores, with a 1-cm^2 cross-sectional area for cooking loss and shear force determinations respectively. The cooking loss, expressed in percentage, was calculated as the difference in the weights before and after cooking divided by the initial weight of the sample, which corresponds to the water loss in the thermal process. The Warner–Bratzler shear force (WBSF) was measured using the conditions described by Madeira et al. (2013).

2.8 | Sensory analysis

Meat sensory characteristics were evaluated by a trained sensory panel in four sessions. The twelve panellists were selected and trained according to Cross et al. (1979). For each session, meat samples were thawed, cooked and prepared according to the study mentioned above. The attributes evaluated were tenderness, juiciness, flavour, off-flavour and overall acceptability, using an eight point-scale, as previously described by Madeira et al. (2013, 2014).

2.9 | Intramuscular fat content and fatty acid composition

Intramuscular fat was extracted from lyophilized samples according to the method of Folch et al. (1957), using dichloromethane–methanol (2:1, v/v) as described by Carlson (1985), and measured gravimetrically after solvent evaporation. Then, intramuscular lipids were transesterified into FAME using a combined basic and acid catalysis, as described by Madeira et al. (2013). FAME were analysed by gas chromatography coupled with flame ionization detection (GC-FID) using a gas chromatograph (HP6890A; Hewlett-Packard) and the same chromatographic conditions as described by Madeira et al. (2014). The quantification of total FAME was done using heneicosaenoic acid (21:0) methyl ester as the internal standard and the conversion of relative peak areas into weight percentages. Results for each fatty acid are expressed as a percentage of the sum of detected fatty acids (% total fatty acids).

2.10 | Determination of β-carotene, total cholesterol, diterpenes and pigments of meat

The simultaneous quantification of total cholesterol, tocopherols and tocotrienols was performed as previously described by Prates et al. (2006). The method involves a direct saponification of the fresh meat, only one *n*-hexane extraction and analysis of the extracted compounds by normal-phase HPLC, using fluorescence (tocopherols and tocotrienols) and UV-visible photodiode array (cholesterol) detections. The contents of total cholesterol, tocopherols and tocotrienols were calculated based on the external standard technique from a standard curve of peak versus compounds concentrations.

The content of chlorophyll *a*, chlorophyll *b*, total carotenoids and total pheophytins was measured according to Teimouri et al. (2013), with slight modifications, as described above for diets.

2.11 | Statistical analysis

All data were checked for normal distribution and variance homogeneity. Data were analysed using the PROC MIXED of SAS software package (version 9.4; SAS Institute Inc.). The model considered the dietary treatment as the single effect. When significant effects of treatments were detected, least-squares means were compared using the PDIFF with the Tukey–Kramer adjustment options of SAS. Results are presented as mean ± *SEM* and were considered significantly different when *p* value was below .05.

3 | RESULTS

3.1 | Intake, growth performance and gastrointestinal tract variables of piglets

Data on intake, growth performance, faecal consistency, TTAD and gastrointestinal tract variables of piglets are presented in Table 3. The experimental diets led to significant differences in piglet weight at the end of the trial ($p < .01$). The control group had higher final weight compared to groups fed with Spirulina, including those supplemented with the exogenous enzymes, with an average difference in weight of 2.8 kg (9.1% of final weight). ADFI was the same for all groups ($p > .05$). ADG was significantly higher for the control group, where piglets grew 96 g/day more than the Spirulina-fed groups. With the same ingestion and lower ADG, the piglets fed with Spirulina diets had higher FCR (1.62, 1.62 and 1.69 for SP, SP + R, SP + L diets respectively) by comparison to the control group (1.48). In addition, the incorporation of exogenous enzymes in diets had no influence on growth, as no significant differences between SP + R and SP + L groups were observed when compared to the SP group ($p > .05$). Also, faecal scores were similar for all groups ($p > .05$).

The incorporation of Spirulina with or without enzyme supplementation affected all TTAD nutritional fractions, with the exception of NDF. TTAD of DM was significantly higher in the control and SP + L groups, with an average difference of 2.1%, by comparison to the other groups. Regarding TTAD of OM, no significant differences were found between the control and the SP and SP + R groups ($p < .05$). Moreover, TTAD of OM for SP + L group was significantly higher than that of the SP + R group. Dietary incorporation of Spirulina, alone or combined with exogenous enzymes, decreased TTAD of CP in 6.6% ($p = .0001$), compared to the control group. TTAD of CF was higher for SP + L group (62.8%), followed by SP + R (60.4%), SP (57.8%) and control (55.6%) groups. TTAD of ADF was

TABLE 3 Effect of diets on feed intake, growth performance, consistency of faeces, total tract apparent digestibility (TTAD) of nutrients and gastrointestinal tract variables of piglets

	Diets				*SEM*	*p* value
	Control	SP	SP + R	SP + L		
Live performance						
Initial weight (kg)	12.1	11.7	12.1	11.9	0.15	.808
Final weight (kg)	31.0^{a}	28.3^{b}	28.4^{b}	27.8^{b}	0.40	.009
ADFI (g)[1]	997	960	943	960	12.8	.521
ADG (g)[2]	677^{a}	593^{b}	582^{b}	567^{b}	12.4	.001
FCR[3]	1.48^{a}	1.62^{b}	1.62^{b}	1.69^{b}	0.023	<.001
Faecal score[4]	0.070	0.223	0.145	0.198	0.032	.355
TTAD (%)						
DM	79.6^{a}	77.6^{b}	77.3^{b}	79.5^{a}	0.35	.014
OM	83.1^{a}	81.3^{b,c}	81.1^{c}	82.9^{a,b}	0.32	.031
CP	80.6^{a}	73.2^{b}	73.4^{b}	75.4^{b}	0.81	<.001
CF	55.6^{a}	57.8^{a,b}	60.4^{b,c}	62.8^{c}	0.78	<.001
NDF	39.8	39.0	37.5	45.4	1.17	.071
ADF	23.0^{a}	28.9^{a,b}	31.1^{a,b}	37.3^{b}	1.86	.039
E	79.9^{a}	78.0^{b}	77.5^{b}	79.9^{a}	0.37	.012
Relative length of gastrointestinal tract (m/kg)						
Small intestine	0.466^{a}	0.487^{a,b}	0.532^{b,c}	0.541^{c}	0.010	.007
Large intestine	0.110	0.122	0.128	0.121	0.003	.154
Content viscosity (cP)						
Duodenum + jejunum	3.16^{a}	4.96^{b}	5.32^{b}	6.11^{b}	0.320	.005
Ileum	5.88^{a}	8.97^{b}	7.77^{a,b}	8.63^{b}	0.403	.023

Note: Dietary treatments: SP—Spirulina diet; SP + R—Spirulina diet supplemented with Rovabio® Excel AP; SP + L—Spirulina diet supplemented with lysozyme.

[1]ADFI—average daily feed intake.

[2]ADG—average daily weight gain.

[3]FCR—feed conversion ratio.

[4]Faecal scores—0 (normal), 1 (soft faeces) or 2 (diarrhoea).

[a,b,c]Values within a row with different superscripts differ significantly at $p < .05$.

TABLE 4 Effect of diets on 24-hr pH, CIE colour parameters (L^*, a^* and b^*) and lipid oxidation evaluated by the concentration of TBARS (mg malonaldehyde/kg of meat) after 0, 3 and 7 days of cold storage of *longissimus lumborum* muscle

	Diets				*SEM*	*p* value
	Control	SP	SP + R	SP + L		
pH 24 hr	5.55	5.50	5.52	5.55	0.062	.902
Colour						
L^*	50.4^{a}	51.5^{a,b}	53.3^{b}	51.9^{a,b}	0.70	.022
a^*	7.08^{a}	7.68^{a,b}	6.99^{a,b}	8.17^{b}	0.263	.008
b^*	0.927^{a}	1.95^{a,b}	2.29^{b}	2.38^{b}	0.264	.001
TBARS[1]						
Day 0	0.233	0.233	0.224	0.245	0.011	.588
Day 3	0.350^{a}	0.814^{b}	0.363^{a,b}	0.546^{a,b}	0.124	.041
Day 7	0.590	1.97	1.09	2.09	0.429	.078

Note: Dietary treatments: SP—Spirulina diet; SP + R—Spirulina diet supplemented with Rovabio® Excel AP; SP + L—Spirulina diet supplemented with lysozyme.

[1]TBARS—Thiobarbituric acid reactive substances.

[a,b]Values within a row with different superscripts differ significantly at $p < .05$.

significantly different between control and SP + L groups, where the SP + L group was 14.3% higher. Finally, TTDA of energy was significantly higher in control and SP + L groups.

Although digesta viscosity of both duodenum plus jejunum ($p = .005$) and ileum ($p = .023$) was higher in Spirulina-fed animals, only the relative length of small intestine in SP + L group was higher ($p < .05$) by comparison to the control and SP groups that had similar values. In particular, piglets fed on SP + L, by comparison to the control group, had a higher increase in viscosity of the duodenum plus jejunum (93%), the ileum (47%) and the relative length of small intestine (16%).

3.2 | pH, colour and lipid oxidation of meat

Meat quality traits assessed in *longissimus lumborum* muscle of piglets are shown in Table 4. Diets had no effect on 24-hr pH values ($p > .05$). Dietary supplementation of exogenous enzymes in Spirulina diets increased meat colour parameters, L^* in SP + R ($p = .022$), a^* in SP + L ($p = .008$) and b^* in both SP + R and SP + L ($p = .001$). In contrast, only a small effect was observed on TBARS values of *longissimus lumborum* muscle at three days of storage at 4°C (Table 4). The incorporation of Spirulina in diet without enzyme supplementation (SP) increased TBARS when compared to the control diet.

3.3 | Cooking loss, shear force and sensory panel scores of meat

The influence of diets on cooking loss, shear force and trained sensory panel scores of *longissimus lumborum* muscle is depicted in Table 5. Neither cooking loss nor shear force was affected by dietary treatments. However, meat from animals fed on SP + L had higher tenderness ($p < .001$) than that from control piglets (4.6 vs. 3.8 respectively). Additionally, when compared to the control group, meat from SP and SP + L groups had higher flavour scores ($p < .05$).

3.4 | Intramuscular fat, total cholesterol and fatty acid composition of meat

Intramuscular fat content and fatty acid profile of *longissimus lumborum* muscle are presented in Table 6. Diets had no impact neither on intramuscular fat and total cholesterol contents nor on major individual fatty acids (18:1*c*9, 23%–26% of total fatty acids; 16:0 and 18:2*n*-6, 20%–21%; and 18:0, 11%), as well as on partial sums and ratios of fatty acids ($p > .05$). Dietary treatment affected only a small number of minor fatty acids, namely 18:3*n*-6, 20:2*n*-6, 20:3*n*-6, 20:3*n*-3, 22:0 and 22:1*n*-9. The incorporation of Spirulina plus enzyme supplementation in the piglet diets increased the percentage of 18:3*n*-6, but decreased the relative proportion of 20:2*n*-6 (both with $p < .001$). When compared to the control diet, the percentages of 20:3*n*-6 were significantly higher in SP groups but more markedly in the groups fed with Spirulina plus enzymes ($p = .016$). Moreover, piglets fed Spirulina diets had higher percentages of 20:3*n*-3 (SP, $p = .001$), 22:0 (SP + R, $p = .027$) and 22:1*n*-9 (SP + L, $p = .037$) than those fed the control diet.

3.5 | Total carotenoids, chlorophylls and vitamin E contents of meat

The effect of diets on total pigments and diterpene profile of *longissimus lumborum* muscle is presented in Table 7. Regarding total pigments, meat of the SP + L group had higher values of chlorophyll *a*, chlorophyll *b*, total chlorophylls, total carotenoids and total pheophytins, comparatively to the control group ($p < .05$). The incorporation of Spirulina in the diets, with or without added enzymes, increased total meat carotenoids by comparison to the control group ($p = .013$). The diterpene profile of meat was similar for all dietary treatments.

4 | DISCUSSION

The incorporation of microalga Spirulina as a feedstuff and the supplementation with two exogenous enzymes, Rovabio® Excel AP

TABLE 5 Effect of diets on cooking loss, shear force and sensory panel scores of *longissimus lumborum* muscle of piglets

	Diets					
	Control	SP	SP + R	SP + L	*SEM*	*p* value
Cooking loss (%)	33.2	32.9	33.0	32.9	0.89	.996
Shear force (kg)	3.79	3.82	3.84	4.10	0.499	.970
Sensory panel scores						
Tenderness	3.76[a]	3.83[a]	4.12[a,b]	4.63[b]	0.159	<.001
Juiciness	4.26	4.27	4.26	4.67	0.128	.050
Flavour	4.08[a]	4.55[b]	4.45[a,b]	4.49[b]	0.110	.012
Off-flavour	0.309	0.344	0.277	0.479	0.106	.529
Overall acceptability	4.15	4.25	4.36	4.63	0.136	.069

Note: Dietary treatments: SP—Spirulina diet; SP + R—Spirulina diet supplemented with Rovabio® Excel AP; SP + L—Spirulina diet supplemented with lysozyme.

[a,b]Values within a row with different superscripts differ significantly at $p < .05$.

TABLE 6 Effect of diets on intramuscular fat content (g/100 g muscle), total cholesterol (mg/100 g muscle) and fatty acid (FA) composition (% of total FA) of *longissimus lumborum* muscle of piglets

	Diets					
	Control	SP	SP + R	SP + L	*SEM*	*p* value
Intramuscular fat	1.07	1.11	1.11	1.16	0.077	.880
Total cholesterol	64.7	64.9	61.2	62.6	2.37	.664
FA composition						
10:0	0.066	0.061	0.068	0.060	0.007	.649
12:0	0.052	0.055	0.066	0.054	0.005	.139
14:0	0.732	0.788	0.787	0.777	0.055	.874
15:0	0.099	0.111	0.103	0.099	0.008	.688
16:0	20.0	21.1	21.1	21.2	0.36	.089
16:1*c*7	0.375	0.312	0.388	0.371	0.021	.067
16:1*c*9	2.00	2.22	2.20	2.03	0.141	.583
17:0	0.515	0.554	0.546	0.516	0.037	.821
17:1*c*9	0.213	0.219	0.232	0.177	0.017	.108
18:0	10.6	10.7	10.8	11.0	0.13	.131
18:1*c*9	25.6	25.3	24.9	23.3	1.19	.510
18:1*c*11	3.32	3.27	3.23	3.11	0.059	.090
18:2*n*-6	20.8	19.8	20.1	21.3	0.89	.600
18:3*n*-6	0.183[a]	0.339[b]	0.339[b]	0.349[b]	0.018	<.001
18:3*n*-3	0.875	0.797	0.833	0.881	0.028	.133
20:0	0.115	0.137	0.108	0.108	0.012	.263
20:1*c*11	0.411	0.404	0.377	0.361	0.025	.450
20:2*n*-6	0.524[a]	0.441[b]	0.447[b]	0.470[b]	0.011	<.001
20:3*n*-6	0.485[a]	0.589[a,b]	0.619[b]	0.630[b]	0.034	.016
20:4*n*-6	4.03	4.05	3.84	4.00	0.359	.973
20:3*n*-3	0.162[a]	0.130[b]	0.140[a,b]	0.153[a]	0.006	.001
20:5*n*-3	0.230	0.210	0.201	0.202	0.023	.780
22:0	0.059[a,b]	0.057[a,b]	0.092[b]	0.032[a]	0.014	.027
22:1*n*-9	0.128[a]	0.144[a,b]	0.170[a,b]	0.205[b]	0.019	.037
22:5*n*-3	0.578	0.583	0.572	0.526	0.067	.920
22:6*n*-3	0.324	0.301	0.289	0.311	0.037	.917
Others	7.37	7.26	7.41	7.76	0.452	.865
Partial sums of FA						
∑SFA[1]	32.3	33.6	33.7	33.8	0.43	.060
∑MUFA[2]	32.1	31.9	31.5	29.6	1.40	.547
∑PUFA[3]	28.2	27.3	27.3	28.8	1.37	.813
∑*n*-3 PUFA[4]	2.17	2.02	2.04	2.07	0.113	.788
∑*n*-6 PUFA[5]	26.1	25.3	25.3	26.8	1.26	.797
Ratios of FA						
PUFA:SFA	0.877	0.817	0.816	0.859	0.506	.771
n-6:*n*-3	12.1	12.6	12.5	12.9	0.28	.302

Note: Dietary treatments: SP—Spirulina diet; SP + R—Spirulina diet supplemented with Rovabio® Excel AP; SP + L—Spirulina diet supplemented with lysozyme.

Abbreviations: MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

[1]Sum of 10:0, 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0 and 22:0.

[2]Sum of 16:1*c*7, 16:1*c*9, 17:1*c*9, 18:1*c*9, 18:1*c*11, 20:1*c*11 and 22:1*n*-9.

[3]Sum of 18:2*n*-6, 18:3*n*-6, 18:3*n*-3, 20:2*n*-6, 20:3*n*-6, 20:4*n*-6, 20:3*n*-3, 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3.

[4]Sum of 18:3*n*-3, 20:3*n*-3, 20:5*n*-3, 22:5*n*-3 and 22:6*n*-3.

[5]Sum of 18:2*n*-6, 18:3*n*-6, 20:2*n*-6, 20:3*n*-6 and 20:4*n*-6.

[a,b]Values within a row with different superscripts differ significantly at $p < .05$.

TABLE 7 Effect of diets on total pigments (μg/g) and diterpene profile (μg/g) of *longissimus lumborum* muscle of piglets

	Diets					
	Control	SP	SP + R	SP + L	*SEM*	*p* value
Pigments						
Chlorophyll *a*[1]	0.221[a]	0.346[a,b]	0.291[a,b]	0.397[b]	0.045	.045
Chlorophyll *b*[2]	0.328[a]	0.508[a,b]	0.475[a,b]	0.653[b]	0.076	.035
Total chlorophylls[3]	0.549[a]	0.853[a,b]	0.766[a,b]	1.05[b]	0.120	.037
Total carotenoids[4]	0.092[a]	0.146[b]	0.150[b]	0.153[b]	0.014	.013
Total chlorophylls and total carotenoids[5]	0.641[a]	0.999[a,b]	0.916[a,b]	1.20[b]	0.132	.033
Diterpene profile						
β-Carotene	0.081	0.081	0.077	0.082	0.003	.668
α-Tocopherol	0.498	0.389	0.504	0.366	0.048	.090
γ-Tocopherol	0.030	0.030	0.031	0.027	0.002	.522

Note: Dietary treatments: SP—Spirulina diet; SP + R—Spirulina diet supplemented with Rovabio® Excel AP; SP + L—Spirulina diet supplemented with lysozyme.

[1]Chlorophyll *a* = 11.24 × A662 nm − 2.04 × A645 nm.

[2]Chlorophyll *b* = 20.13 × A645 nm − 4.19 × A662 nm.

[3]Total chlorophylls (C*a* + *b*) = 7.05 × A662 nm + 18.09 × A645 nm.

[4]Total carotenoids (Cx + *c*) = (1,000 × A470 nm − 1.90 × Ca − 63.14 × Cb)/214.

[5]Total chlorophylls and carotenoids = (Ca + *b*) + (Cx + *c*).

[a,b]Values within a row with different superscripts differ significantly at $p < .05$.

and lysozyme, was the focus of this study. There are several studies about the incorporation of Spirulina in piglet feeding as a supplement (Furbeyre et al., 2017; Grinstead et al., 2000; Nedeva et al., 2014), but none using it as an ingredient.

The incorporation of 10% Spirulina affected negatively growth performance, with a 9.1% decrease on final weight, a 14.2% decrease on ADG and a 11.0% increase on FCR, relatively to the control group. All diets used in this study provide required CP and total essential amino acids levels according to the specific requirements of the species (NRC, 2012). Therefore, no deficit in these nutrients was expected, so as to justify the lower growth performance of piglets receiving Spirulina diets. As a supplement, Neveda et al. (2014) tested the inclusion of 0.15% and 0.2% of Spirulina to piglet diets (from 12.2–12.5 to 30.9–33.9 kg live weight) and found a significant increase on growth, while feed conversion ratio decreased. Conversely, Grinstead et al. (2000) studied the zootechnical parameters in weaned piglets fed 0.2%, 0.5% and 2% of Spirulina for 28 days and the results were inconsistent, with minimal improvement on growth performance. This was possibly due to the circumstance that these two studies considered the incorporation of Spirulina only as a supplement, in incorporation levels not high enough to cause a negative effect on growth performance of piglets, as observed in our study. However, Simkus et al. (2013) reported that ADG in fattening pigs fed Spirulina may increase up to 15%–26%, with no effect on back-fat thickness. The supplementation with the exogenous enzymes did not improve growth performance because no differences were detected between SP, SP + R and SP + L dietary groups.

The results of TTAD for CP herein reported clearly indicate that microalgae proteins, at this incorporation level, were not extensively and adequately digested by the piglets. These results, and the lower TTAD for E on diets SP and SP + L, are in agreement with the piglet's growth performance as the obtained values were higher for the control group, by comparison to the others. It is noticeable that the SP + L group had comparable (DM and E TTAD) or higher (CF and ADF TTAD) values than the ones observed in the control group. Comparing the SP and SP + L groups, the improvement of TTAD of DM in the SP + L group is justified by the increase in TTAD of CF, ADF and E. This indicates that lysozyme was effective in degrading the Spirulina cell wall, thus facilitating the access of digestive enzymes to the cell content. However, Spirulina's protein fraction seems to be resistant to the action of piglet endogenous peptidases, thus justifying the lower TTAD of CP to SP, SP + R and SP + L groups. Moreover, the fermentation of Spirulina cell wall in the hindgut may increase the excretion of bacterial protein in faeces and reduce the TTAD of CP (Schulze et al., 1995).

Lower protein digestibility is associated with higher digesta viscosity, which limits the access of the endogenous enzymes to their target substrates. In turn, the higher digesta viscosity observed in piglets receiving Spirulina led to a compensatory small intestine enlargement. In fact, results indicate that the increase in digesta viscosity is not a consequence of the presence of soluble polysaccharides, such as arabinoxylans and β-glucans, since the presence of xylanases and β-glucanases in the SP + R group had no effect on viscosity. Thus, the high increase in digesta viscosity of piglets fed with microalga is likely a result of gelation of the low-digestible Spirulina proteins, as suggested by Evans et al. (2015). In fact, these authors suggested that when Spirulina is incorporated in poultry diets at high levels (>10%), it is observed a gelation of its proteins,

which contributes to increased digesta viscosity and reduced amino acid digestibility. The degradation of Spirulina cell wall by lysozyme, in piglets of the SP + L group, led to a higher release of microalga proteins, increasing digesta viscosity, but also to a higher ADF degradation, decreasing digesta viscosity. Overall, data suggest that a high proportion of Spirulina proteins are resistant to the proteolytic action of piglet endogenous peptidases.

Regarding meat colour parameters, groups fed with Spirulina and exogenous enzymes had a lighter, redder and yellower meat than the meat of the group fed with the control diet. In addition, meat from SP and SP + L groups had higher scores of tenderness and flavour, indicating that Spirulina incorporation had no negative effect on meat flavour.

TBARS values of *longissimus lumborum* muscle at 3 days of storage at 4°C were higher for SP group, by 32.6%, in comparison with the control group. The results on the oxidative stability of meat did not reflect the antioxidant activity of Spirulina, reported by some authors (Gutiérrez-Salmeán et al., 2015; Hoseini et al., 2013), because no protective effect against lipid oxidation was observed in pork during the 7 days of storage. However, the higher content of antioxidant pigments in diets incorporated with Spirulina, like β-carotene, would lead us to predict a noticeable effect on meat antioxidants. In fact, this content of β-carotene was not significantly different in meat (Table 7). Moreover, the major individual fatty acids were not affected by the diets. The higher concentrations of 18:3*n*-6 observed in meat of Spirulina groups can putatively be explained by its predominance in Spirulina, as previously suggested elsewhere (Gutiérrez-Salmeán et al., 2015).

Concerning pigment contents, the meat of SP + L group had an increase of 79.6%, 99.1%, 91.4%, 87.8% and 107% regarding chlorophyll *a*, chlorophyll *b*, total chlorophylls, total carotenoids and total pheophytins, respectively, and by comparison to the control group. SP + L diet had the higher pigment contents when compared to the other diets, which could possibly explain such an increase observed in meat. By comparison to the control group, meat of the other groups had an average increase of 62.7% in total carotenoids. This is related to the fact that these diets had five times more carotenoids than the control diet.

Simkus et al. (2013) reported that crossbreds of Landrace and Yorkshire fattening pigs fed daily 2 g of 75% humidity fresh Spirulina had no effect on pork traits, such as colour, pH, cooking loss and tenderness. Similarly, in our experiment most of the pork traits were also not affected by the Spirulina incorporation, indicating that meat of pigs fed with Spirulina has similar properties to those of animals fed on standard diets, such as the control used in this study.

5 | CONCLUSIONS

The results in this study clearly show, for the first time, the feasibility of the use of Spirulina as an alternative feedstuff in piglet feeding. Nevertheless, there are clear losses in production parameters by comparison to diets including standard proteinaceous feedstuffs. Indeed, the study indicates that growth performance of post-weaning piglets was diminished by the incorporation of 10% Spirulina in diets. Such decrease in animal performance was due to the low digestibility and gelation of Spirulina proteins in the intestine, as a direct consequence of their proteolytic resistance to the piglet endogenous peptidases. In addition, the use of carbohydrases in the feed does not improve the digestive utilization of this microalga by piglets, in our experimental conditions. However, the effectiveness of lysozyme in the degradation of Spirulina cell wall in the piglet's intestine, with the consequent liberation of nutritional compounds, is an important outcome of this work. This finding warrants further studies that may ultimately contribute to a better digestibility of dietary Spirulina. In general, meat quality traits are not negatively affected by the addition of Spirulina, alone and combined with enzymes, to the piglet's feeding.

Further research should be conducted to assess the effect of exogenous peptidases (EC 3.4), most likely from marine organisms, to improve the digestibility of proteins of this microalga. In line with this, in vitro studies will be performed in the near future to find the best endopeptidase candidate to degrade Spirulina proteins and avoid their gelation. Later on, the supplementation of piglet diets with the selected endopeptidase, combined or not with lysozyme, will be tested in order to make effective the use of Spirulina in swine nutrition.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

ANIMAL WELFARE STATEMENT

All the procedures used in this animal experiment were reviewed by the Ethics Commission of ISA and approved by the Animal Care Committee of the National Veterinary Authority (Direção Geral de Alimentação e Veterinária, Portugal), following the principles and specific guidelines of the European Union legislation (2010/63/EU Directive).

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