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Influence of dietary Spirulina inclusion and lysozyme supplementation on the *longissimus lumborum* muscle proteome of newly weaned piglets

David M. Ribeiro [a,1], Cátia F. Martins [a,b,1], Josipa Kuleš [c], Anita Horvatić [c,d], Nicolas Guillemin [c], João P.B. Freire [a], P. David Eckersall [c,e], André M. Almeida [a,*,2], José A.M. Prates [b,2]

[a] LEAF - Linking Landscape, Environment, Agriculture and Food, Instituto Superior de Agronomia, Universidade de Lisboa, 1349-017 Lisboa, Portugal
[b] CIISA – Centro de Investigação Interdisciplinar em Sanidade Animal, Faculdade de Medicina Veterinária, Universidade de Lisboa, 1300-477 Lisboa, Portugal
[c] Laboratory of Proteomics, Internal Diseases Clinic, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10000 Zagreb, Croatia
[d] Department of Chemistry and Biochemistry, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia
[e] Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medicine, Veterinary Medicine and Life Sciences, University of Glasgow, Glasgow G61 1QH, UK

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ABSTRACT

Arthrospira platensis (Spirulina) is a microalga with a high content of crude protein. It has a recalcitrant cell wall that limits the accessibility of the animal endogenous enzymes to its intracellular nutrients. Enzymatic supplementation aiming to degrade cell walls could benefit microalgae digestibility. The objective of this study was to evaluate the impact of dietary Spirulina and lysozyme supplementation over the muscle proteome of piglets during the post-weaning stage. Thirty piglets were randomly distributed among three diets: control (no microalga), SP (10% Spirulina) and SP + L (10% Spirulina +0.01% lysozyme). After 4 weeks, they were sacrificed and samples of the *longissimus lumborum* muscle were taken. The muscle proteome was analysed using a Tandem Mass Tag (TMT)-based quantitative approach. A total of 832 proteins were identified. Three comparisons were computed: SP vs Ctrl, SP + L vs Ctrl and SP + L vs SP. They had ten, four and twelve differentially abundant proteins. Glycogen metabolism and nutrient reserves utilization are increased in the SP piglets. Structural muscle protein synthesis increased, causing higher energy requirements in SP + L piglets. Our results demonstrate the usefulness of proteomics to disclose the effect of dietary microalgae, whilst unveiling putative mechanisms derived from lysozyme supplementation. Data available via ProteomeXchange with identifier PXD024083.
Significance: Spirulina, a microalga, is an alternative to conventional crops which could enhance the environmental sustainability of animal production. Due to its recalcitrant cell wall, its use requires additional measures to prevent anti-nutritional effects on the feeding of piglets in the post-weaning period, during which they endure post-weaning stress. One of such measures could be CAZyme supplementation to help degrade the cell wall during digestion. Muscle proteomics provides insightful data on the effect of dietary microalgae and enzyme activity on piglet metabolism.

1. Introduction

Global population is rapidly increasing, as are living standards, which are expected to greatly increase the demand and price of animal products, such as meat or eggs [1]. Maize and soybean meal (SBM) are extensively used in monogastric animal diets around the world [2]. Consequently, the demand for these conventional feedstuffs will likely increase in parallel to that of animal products. Soybean meal and maize are essentially produced in American countries, like Argentina, Brazil and the United States of America [3], and are exported to major markets for animal production, namely China and the European Union. The production of these crops raises questions on its sustainability [4], particularly given the high transportation costs and resulting greenhouse gas emissions. Therefore, it is important to find sustainable and viable alternatives that can contribute towards enhanced animal production and hence replace such feedstuffs. Microalgae are increasingly

* Corresponding author.
E-mail address: aalmeida@isa.ulisboa.pt (A.M. Almeida).
[1] Both authors contributed equally to this paper and should be considered co-authors.
[2] André Martinho de Almeida and José António Mestre Prates have equal contributions and are joint senior authors.

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considered as an interesting alternative [2].

Microalgae, a large group of uni- or multi-celular organisms, are divided into 4 major classes: diatoms (*Bacillarophyceae*), green (*Chlorophyceae*), golden (*Chrysophyceae*) and blue-green (*Cyanophyceae*) algae [2]. Spirulina, *Arthrospira platensis*, belongs to the latter. It has a particularly high crude protein (CP) content (62–72% in dry matter – DM) and up to 44.2% poly-unsaturated fatty acids (PUFA) in total fatty acids [1], which makes it a potentially viable alternative to proteinaceous feedstuffs such as SBM. However, like other microalgae such as *Chlorella vulgaris* [5], it poses several challenges when included in monogastric diets. Indeed, and due to its recalcitrant cell wall [6], nutrient availability is very limited. Therefore, animal nutritionists should establish strategies to improve nutrient digestibility when formulating diets including the raw biomass of microalgae. One of such strategies is the supplementation of carbohydrate-active enzymes (CAZymes) to promote the degradation of the cell wall [6].

Piglet nutrition is a very important field within the pig industry. Adequate *pre*-weaning nutrition guarantees the full development of the piglet, whilst promoting the intake of solid feed for a better adaptation to the *post*-weaning period [7]. This is particularly important in Mediterranean countries, given the demand for suckling piglets to produce roasted piglet traditional products, such as *Leitão da Bairrada* in Portugal, *Cochinillo Asado* in Spain and *Porcheddu* in Italy [8,9]. The production of these regional dishes often depends on autochthonous breeds, such as the Portuguese *Bísaro*. Around 70% of weaned piglets of this breed are slaughtered for this purpose [9]. The post-weaning period is very critical. *Post*-weaning stress (PWS) is associated with the adaptation to nutritional, social and environmental changes, often accompanied with decreased growth and immune depression, where issues such as diarrhoea have also a significant detrimental effect [10]. To deal with this period, high-quality feeds are required. Current literature presents several results where the incorporation of small levels of microalgae improves animal performance, intestinal structure, and feed conversion, due to the promotion of beneficial bacteria in the gut, such as *Lactobacillus* [1]. Higher levels of incorporation, to supply major nutrients, have been reported with defatted microalgae biomass [11,12]. Ekmay et al. [12] reported that the use of proteolytic enzymes improves the final weight of piglets fed with 10% *Desmodesmus* sp. defatted biomass compared to piglets of control. Our team, in a recent paper, reported that feeding piglets with 10% whole Spirulina, regardless of enzyme supplementation, lowered growth performance, averaging 9.1% decrease in final weight, compared to the control. Lysozyme, despite achieving the degradation of the cell wall, did not improve the digestibility of Spirulina, because of the resistance of its protein against endogenous digestive enzymes, which increases digesta viscosity [8]. Interestingly, knowledge on the physiological impact of the use of dietary microalgae in pig tissues proteome is absent from available literature.

Omics (genomics, transcriptomics, proteomics and metabolomics) technologies are approaches that enable the study of tissue metabolism at the molecular level, as affected by a pre-determined factor. Proteomics allows the study of the proteome, which is the group of proteins present in a given tissue at a given time point. Farm animal proteomics in particular, has gained increased attention due to its numerous applications, from improving reproduction/selection programmes to product quality and traceability [13,14]. Pig production has benefited from this technology [15,16]. Using omics to study complex physiological aspects, such as stress response in pigs, has allowed the discovery of several pathways and putative biomarkers indicating stress susceptibility or resilience [16]. Biomarkers, that can be established using proteomics, have the potential to introduce a valuable feedback system, in which the practices of the several production levels (reproduction, feeding, slaughter, etc.) can be monitored and evaluated to improve decision making [17]. Recent studies published using proteomics in pigs fed with different protein levels [18], under protein restriction [19] and different diet composition [20] clearly demonstrate the interest of this tool in swine nutrition and physiology. To the best of our knowledge, no study has been published on the effect of dietary microalgae on the proteome of any swine tissue. Indeed, and to our knowledge, such an approach was only conducted by our research team in a recent study on the effect of *Nannochloropsis oceanica* dietary inclusion on the rabbit muscle proteome [21].

Recent literature lacks the evaluation of the impact of dietary factors on pig tissue proteomes. Those that do study this subject, do not focus on either Spirulina (or microalgae for that matter) or CAZyme dietary inclusion as influencing factors. It is our perspective that the potential for the use of proteomics in the field of pig nutrition is immense. With it, we can analyse dietary effects at the molecular level: nutrient metabolism, mechanisms of adaptation to detrimental feedstuffs/restrictions, among many other approaches. When studying novel feedstuffs, scientists benefit from the use of these state-of-the-art technologies. These allow an in-depth characterization of their impact on animal metabolism, which goes beyond the analysis of classical data (such as digestibility coefficients). In fact, it allows studying the effect after nutrient digestibility and how the animal in question copes with it. Thus, the objective of this study was to evaluate the effect of dietary Spirulina and lysozyme supplementation on the *longissimus lumborum* muscle proteome of recently weaned piglets.

2. Materials and methods

2.1. Animals and diets

The procedures describing animals and experimental conditions have been reported elsewhere [8]. Briefly, 30 commercial male piglets ([Large White × Landrace] × Pietrain), weaned at 28 days of age and with an average live weight (LW) of 12.0 ± 0.89 kg were evenly distributed in pens to form three groups ($n = 10$). Each group received a different experimental diet: control, control with 10% Spirulina (SP) and control with 10% Spirulina supplemented with 0.01% of lysozyme (Sigma 62,971, Sigma-Aldrich Ltd. St. Louis, MO, USA – SP + L). The composition of experimental diets was published previously and is shown for contextual reasons in Supplementary Table 1. For a period of 28 days, feed and water was available *ad libitum* and animals were weighed weekly. At the end of the experimental period, animals were unsensitised by electrical stunning and slaughtered by exsanguination, following standard procedures of commercial abattoirs. Samples of *longissimus lumborum* muscle from each experimental group were removed from the left side of the carcass following the last thoracic vertebrae. They were subsequently frozen and stored at −80 °C until further analysis.

2.2. Animal welfare statement

All procedures used were reviewed by the Ethics Committee of the Instituto Superior de Agronomia and approved by the Animal Care Committee of the National Veterinary Authority (Direção Geral de Alimentação e Veterinária, Lisbon, Portugal): reference 0421/000/000/2017, date 30-06-2017, following the specific guidelines of national and European Union legislation (Directive 2010/63/EU).

2.3. Protein extraction

Five muscle samples per experimental group were randomly selected and used for proteomic analysis. Cold cut frozen muscle samples were homogenized in 500 ml lysis buffer (100 mM TEAB, 2% SDS) using an Omni TH220 homogenizer (Omni International, Kennesaw, USA), followed by 2 cycles of sonication at maximum amplitude (Qsonica, Newtown, USA) on ice. After centrifugation at 16000 ×*g* at 4 °C for 30 min, clarified supernatant was transferred into a new tube.

2.4. Protein identification and quantification using TMT approach

Sample extracts from each group were processed using the filter aided sample preparation (FASP) protocol and Tandem Mass Tag (TMT)-based quantitative approach. In brief, total protein concentration was determined using BCA assay (Thermo Scientific, Rockford, USA). For each sample and internal standard (pool of all samples), an amount of 35 μg of total proteins was diluted to a volume of 200 μl using urea buffer (8 M urea in 0.1 M Tris–HCl pH 8.5) and subjected to the FASP protocol with some modifications [22]. Samples were transferred to the 10-kDa membrane filter units (Microcon YM-10, Merck Millipore), centrifuged (13,000 ×*g*, 20 min, 20 °C) and washed subsequently with 200 μl of FASP-urea buffer. Proteins were alkylated (50 mM IAA, 20 min at room temperature in the dark), washed twice with urea buffer and then twice with triethyl ammonium bicarbonate (100 mM pH 8.5) (TEAB, Thermo Scientific, Rockford, USA) followed by centrifugation. Proteins were digested by trypsin gold (Promega, enzyme-to-protein ratio 1:35, v/v, at 37 °C overnight). Peptides were then collected from filter by centrifugation, washed with 50 μl of TEAB/ACN (1:1, v/v), centrifuged and vacuum dried. TMT tenplex reagents (Thermo Scientific, Rockford, IL, USA) for muscle samples were prepared according to manufacturer's procedure, as described previously [23]. An amount of 19 μl of specific TMT label was added to each sample for labelling (60 min, room temperature). The reaction was quenched using 5% hydroxylamine (Sigma-Aldrich, St. Louis, MO, USA). Nine TMT-modified samples were randomly combined with the internal standard (labelled with TMT *m/z* 126), aliquoted, dried and stored at −20 °C for further analysis. In total, two tenplexes were prepared for muscle samples.

High resolution LC-MS/MS analysis of TMT-labelled peptides was carried out using an Ultimate 3000 RSLCnano system (Dionex, Germering, Germany) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides were dissolved in loading solvent (1% ACN, 0.1% formic acid) and loaded onto the trap column (C18 PepMap100, 5 μm, 100A, 300 μm × 5 mm), desalted for 12 min at the flow rate of 15 μl/min and separated on the analytical column (PepMap™ RSLC C18, 50 cm × 75 μm) using a linear gradient of 5–45% mobile phase B (0.1% formic acid in 80% ACN) over 120 min, 45% to 90% for 2 min, held at 80% for 2 min and re-equilibrated at 5% B for 20 min at the flow rate of 300 nl/min. Mobile phase A consisted of 0.1% formic acid in water. Ionisation was achieved using nanospray Flex ion source (Thermo Fisher Scientific, Bremen, Germany) containing a 10 μm-inner diameter SilicaTip emitter (New Objective, USA). The MS operated in positive ion mode using DDA Top8 method. Full scan MS spectra were acquired in range from *m/z* 350.0 to m/z 1800.0 with a resolution of 70,000, 110 ms injection time, AGC target 1 × E6, a ± 2.0 Da isolation window and the dynamic exclusion 30 s. HCD fragmentation was performed at step collision energy (29% and 35% NCE) with a resolution of 17,500 and AGC target of 2 × E5. Precursor ions with unassigned charge state, as well as charge states of +1 and more than +7 were excluded from fragmentation.

Acquired MS/MS spectra were analysed for protein identification and quantification using the SEQUEST algorithm implemented into Proteome Discoverer (version 2.0., ThermoFisher Scientific). Database search against *Sus scrofa* FASTA files (downloaded from Uniprot database 04/18/2018, 77,933 entries) was performed according to the following parameters: two trypsin missed cleavage sites, precursor and fragment mass tolerances of 10 ppm and 0.02 Da, respectively; carbamidomethyl (C) fixed peptide modification, oxidation (M), deamidation (N,Q) and TMT sixplex (K, peptide N-terminus) dynamic modifications. The false discovery rate (FDR) for peptide identification was calculated using the Percolator algorithm in the Proteome Discoverer workflow based on the search results against a decoy database and was set at 1% FDR. At least two unique peptides and 5% FDR were required for reporting confidently identified proteins.

Protein quantification was accomplished by correlating the relative intensities of reporter ions extracted from tandem mass spectra to that of the peptides selected for MS/MS fragmentation. The internal standard was used to compare relative quantification results for each protein between the experiments.

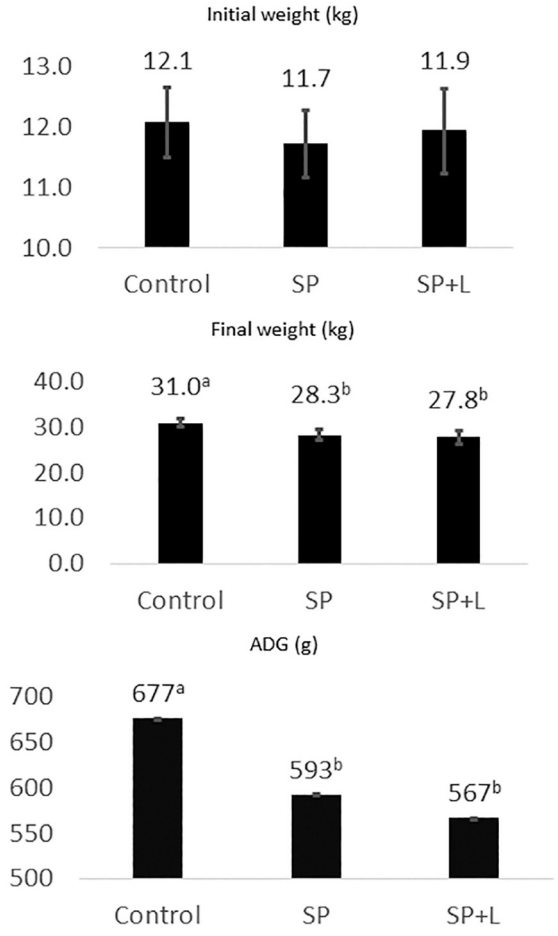

Fig. 1. Initial weight, final weight, and average daily gain (ADG) of piglets fed with the different dietary treatments: control, SP (10% Spirulina) and SP + L (10% Spirulina + 0.01% lysozyme). Means with different superscripts are significantly different ($p < 0.05$). Statistical analysis performed elsewhere [8].

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [23] partner repository with the dataset identifier PXD024083.

2.5. Statistical analysis

Statistical analysis followed the procedures reported elsewhere [24–26]. All statistical analyses were carried out using the R software, under the R Studio environment [27]. A Wilcoxon test was performed to detect significant differences using the stats package. Fold change for each group comparison was obtained by using the log2(Mean A/Mean B) function. When $p < 0.05$, proteins were considered significantly different between comparisons. Gene ontology classification was performed using PANTHER [28].

3. Results

3.1. Animal performance

Animal performance and feed digestibility were previously reported [8] and are briefly presented in this work for contextual purposes only. Fig. 1 depicts the performance results obtained. Overall, the control group had statistically significant higher final weight compared to the other groups. The same relation was found for average daily gain (ADG).

Table 1
Differentially abundant proteins for SP vs control comparison in the *longissimus lumborum* muscle.

Accession number	Protein description [*Sus scrofa*]	p-value	Fold change
A0A287BNL5	Uncharacterized protein OS=*Sus scrofa* OX = 9823 PE = 4 SV = 1	0.01587	−0.70321
F1SS62	Myosin heavy chain 8 OS=*Sus scrofa* OX = 9823 GN = MYH8 PE = 3 SV = 3	0.01587	−0.54227
Q19PY2	Alpha-1,4 glucan phosphorylase OS=*Sus scrofa* OX = 9823 GN=PYGM PE = 2 SV = 2	0.01945	−0.46302
F1S557	Amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase OS=*Sus scrofa* OX = 9823 GN = AGL PE = 1 SV = 2	0.02940	0.05859
Q29568	Phosphopyruvate hydratase (Fragment) OS=*Sus scrofa* OX = 9823 PE = 2 SV = 1	0.04909	0.11969
A0A287B441	ATP synthase-coupling factor 6, mitochondrial OS=*Sus scrofa* OX = 9823 GN = ATP5J PE = 3 SV = 1	0.01945	0.15757
F1S156	Synaptopodin 2 OS=*Sus scrofa* OX = 9823 GN=SYNPO2 PE = 1 SV = 3	0.01565	0.21333
H6TBN0	Thioredoxin OS=*Sus scrofa* OX = 9823 GN = TRX1 PE = 2 SV = 1	0.02843	0.23739
G9F6X8	Protein disulfide-isomerase OS=*Sus scrofa* OX = 9823 GN=P4HB PE = 2 SV = 1	0.01945	0.33956
A0A287BF33	Actin, alpha skeletal muscle OS=*Sus scrofa* OX = 9823 GN = ACTA1 PE = 1 SV = 1	0.01587	0.45446

3.2. Proteomic analysis

A total of 832 proteins were successfully identified and quantified in the piglet *longissimus lumborum* muscle (Supplementary File 1). Three comparisons were made between all experimental groups (Table 1, Fig. 2A). Between SP and control groups, ten proteins were identified with differential abundance. The 10 proteins more abundant in the SP group were mostly involved in catalysis (GO:0003824). These include amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase (AGL), ATP synthase-coupling factor 6 (ATP5J) and protein disulphide-isomerase (P4HB). In contrast, the control group had 4 highly abundant proteins, such as myosin heavy chain 8 (MYM8) and alpha-1,4 glucan phosphorylase (PYGM).

The SP + L vs Control comparison yielded the lowest number of differentially abundant proteins (Table 2, Fig. 2B). The SP + L group had increased accumulation of apolipoprotein A-I (APOA1), whereas the control had higher accumulation of L-lactate dehydrogenase (LDH) and thioredoxin (TRX1).

Finally, the highest number of differentially abundant proteins were obtained in the SP + L vs SP comparison (Table 3, Fig. 2C). The latter group has higher abundance of four proteins, such as caveolae associated protein 1 (CAVIN1) and alpha-1,4 glucan phosphorylase (PYGM), which have mostly catalytic activity (GO:0003824). In turn, the SP + L group has higher abundance of structural/contractile apparatus proteins such as tropomyosin 3 (TPM3) and myosin light chains (MYL2, MYL3, MYL10). It also had higher abundance of aconitate hydratase (ACO2).

All results regarding the gene ontology classification of differentially abundant proteins are available in Fig. 3.

4. Discussion

Using proteomics to evaluate animal metabolism in nutrition-oriented studies has been extensively reported in recent years. However, to our knowledge, the specific evaluation of the inclusion of microalgae effect over pig muscle proteome remains to be established. Recently, the effect of dietary *Nannochloropsis oceanica* on the rabbit muscle proteome has been described [21]. The authors found that 4.45% microalga inclusion affected muscle amino acid metabolism and protein turnover, which is possibly related with the recalcitrant cell wall of microalgae. Spirulina, used in the present study, currently lacks a similar analysis. The effect of external enzymes to improve its nutrient availability also warrants further discussion.

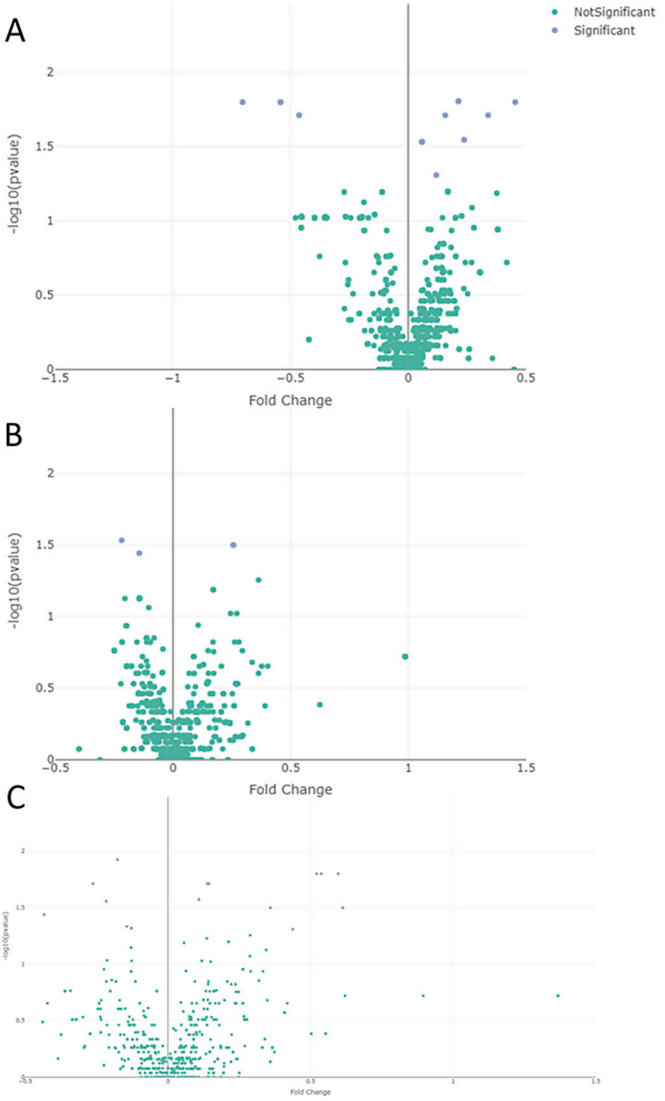

Fig. 2. Volcano plots obtained for each comparison: SP vs Ctrl (A), SP + L vs Ctrl (B) and SP + L vs SP (C). Plots the fold changes of each protein (x-axis) with the p-value (y-axis) obtained from the Wilcoxon test. Significantly different proteins are marked in purple, non-significant are marked in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Differentially abundant proteins for SP + L vs control comparison in the *longissimus lumborum* muscle.

Accession number	Protein description [Sus scrofa]	p-value	Fold change
D2SW96	L-lactate dehydrogenase OS=*Sus scrofa* OX = 9823 PE = 2 SV = 1	0.03615	−0.14336
K7GM40	Apolipoprotein A-I OS=*Sus scrofa* OX = 9823 GN = APOA1 PE = 3 SV = 2	0.03175	0.25627
H6TBN0	Thioredoxin OS=*Sus scrofa* OX = 9823 GN = TRX1 PE = 2 SV = 1	0.02940	−0.21798

Table 3
Differentially abundant proteins for SP + L vs SP comparison in the *longissimus lumborum* muscle.

Accession number	Protein description [Sus scrofa]	*P* value	Fold Change
Q19PY2	Alpha-1,4 glucan phosphorylase OS=*Sus scrofa* OX = 9823 GN=PYGM PE = 2 SV = 2	0.037	−0.43368
A0A2C9F3B2	Voltage-dependent anion-selective channel protein 1 OS=*Sus scrofa* OX = 9823 GN=VDAC1 PE = 1 SV = 1	0.019	−0.26265
F1SLF0	Uncharacterized protein OS=*Sus scrofa* OX = 9823 GN=SPR PE = 1 SV = 3	0.012	−0.17722
A0A287A2G4	Caveolae associated protein 1 OS=*Sus scrofa* OX = 9823 GN=CAVIN1 PE = 4 SV = 1	0.048	−0.12808
Q0QF26	Malate dehydrogenase (Fragment) OS=*Sus scrofa* OX = 9823 GN = MDH2 PE = 2 SV = 1	0.027	0.10852
F1SRC5	Aconitate hydratase, mitochondrial OS=*Sus scrofa* OX = 9823 GN = ACO2 PE = 1 SV = 3	0.019	0.14414
Q2XQY5	Tropomyosin 3 OS=*Sus scrofa* OX = 9823 GN = tpm3 PE = 2 SV = 1	0.032	0.35930
A0A287AIU7	Troponin C, slow skeletal and cardiac muscles OS=*Sus scrofa* OX = 9823 GN = TNNC1 PE = 1 SV = 1	0.049	0.43762
A1XQV9	MLC2v (Fragment) OS=*Sus scrofa* OX = 9823 GN = MLC2V PE = 2 SV = 1	0.016	0.52135
Q8MHY0	MYL2 OS=*Sus scrofa* OX = 9823 GN = MLC-2 V PE = 1 SV = 1	0.016	0.53751
F1RKG0	Myosin light chain 10 OS=*Sus scrofa* OX = 9823 GN = MYL10 PE = 4 SV = 3	0.016	0.59674
K4EJ64	MYL3 OS=*Sus scrofa* OX = 9823 GN = MYL3 PE = 2 SV = 1	0.032	0.61319

4.1. The effect of dietary Spirulina on the piglet muscle proteome

In the comparison of SP vs Control, two different contractile apparatus proteins were detected with differential abundance: myosin heavy chain 8 (MYH8) and alpha actin (ACTA1), with higher abundance in control and SP groups, respectively. The former takes part in actin filament binding, whereas the latter is acted upon by myosin during muscle fibre contraction. It is furthermore involved in cell organization [29]. It has been reported that farm animals under weight loss [30,31] have lower abundance of structural proteins compared to growing animals as a consequence of lower muscle development. A similar reasoning could be considered for this study. Indeed, CP digestibility and growth were lower in the groups fed with microalgae, compared to control [8]. However, since both groups had higher abundance of two different contractile apparatus proteins, these differences could reflect differential organization of the contractile process. Indeed, Xu et al. [32] found that Meishan pigs had higher abundance of ACTA1 (by comparison to Large White) in the skeletal muscle, indicating increased need of actin to promote myofibril assembly. Moreover, Fernández-Barroso et al. [29] found via a transcriptomics approach, that both MYH8 and ACTA1 genes are overexpressed in Iberian pigs with tougher meat. We found no differences regarding shear force between control and SP groups [8]. The reason(s) why microalgae inclusion causes these differences are unclear and require further investigation.

Piglets fed with 10% Spirulina had a higher abundance of glycogen debranching enzyme (GAL) which, along with glycogen phosphorylase (PYGM), are responsible for the degradation of glycogen [33]. The combined action of these enzymes generates glucose-1-P, negatively charged and unable to diffuse out of the muscle cells, where it remains available for conversion into glucose-6-P by phosphoglucomutase [33]. Glucose-6-P is then available to enter the glycolysis pathway. Interestingly, the SP group also had higher abundance of phosphopyruvate hydratase (ENO1), which acts in the glycolysis pathway, converting 2-phosphoglycerate into phosphoenolpyruvate. This set of reactions

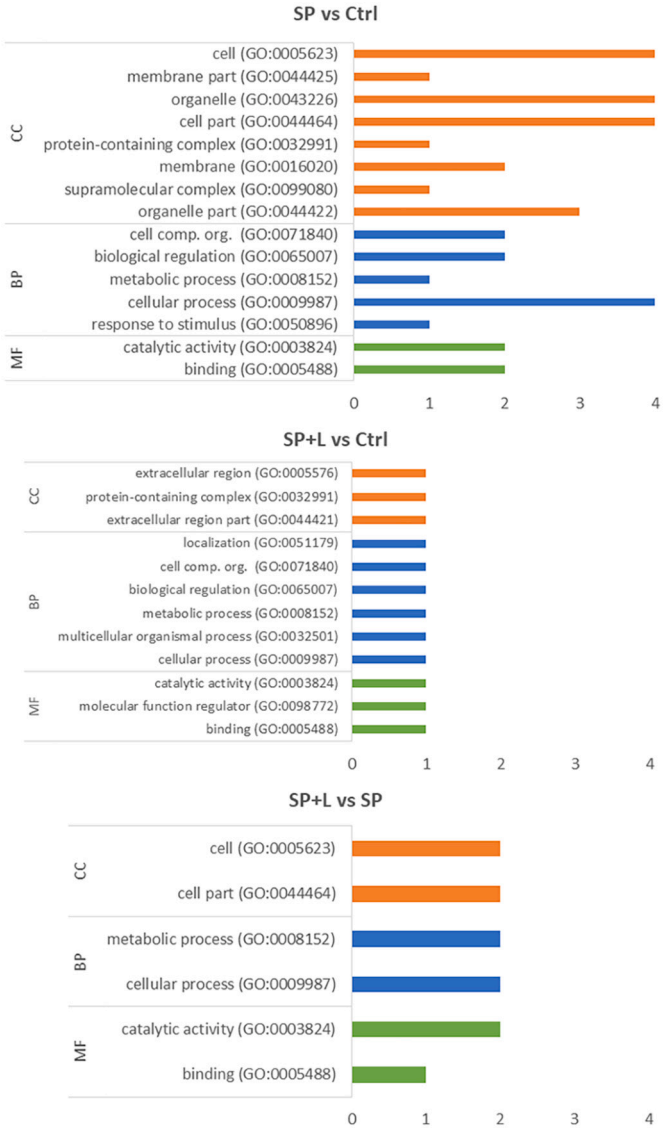

Fig. 3. Cellular components (CC - orange), biological processes (BP - blue) and molecular functions (MF - green) of proteins identified in all comparisons of the muscle proteome of piglets. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

generates high yields of pyruvate, which becomes available for ATP production via the tricarboxylic acid (TCA) cycle. Finally, this same group of piglets had higher abundance of ATP-synthase-coupling factor 6 (ATP5J), which produces ATP from ADP in the presence of a proton gradient. Overall, these results point towards the depletion of higher energy reserves in the SP group compared to the control group. This could derive from the lower nutrient digestibility, as mentioned earlier.

Control piglets had higher abundance of alpha- 1,4 glucan phosphorylase (PYGM) compared to SP. This protein is part of the glycogenolysis pathway in tandem with GAL, when activated via phosphorylation [34]. This process is of paramount importance to the development of desirable meat quality traits post-mortem [35]. However, the higher abundance of PYGM in control piglets likely derives from their higher muscle development. It has been reported by Xu et al. [36] that PYGM is elevated during pig skeletal muscle development. In addition, the authors found higher PYGM mRNA levels in Large White vs Meishan muscle. The former has higher muscle development, characteristic of western breeds. This information is in accordance with our results regarding animal performance, as the final weight and average

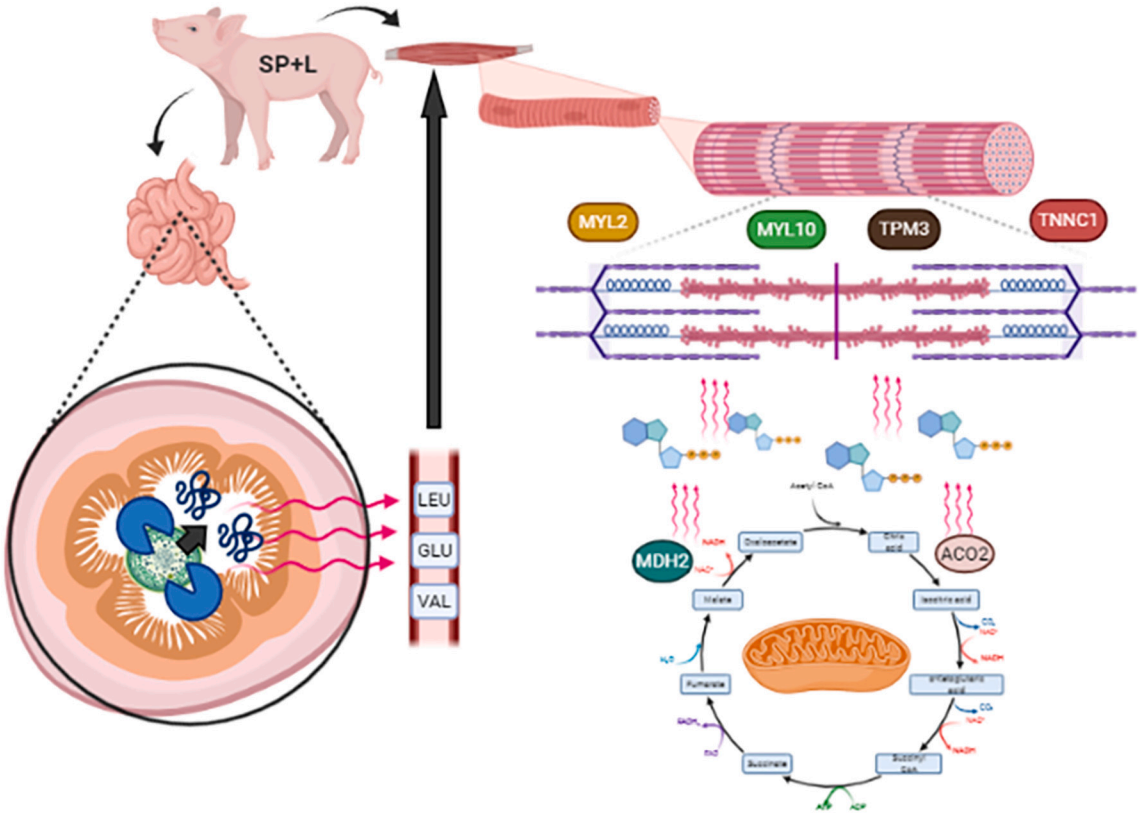

Fig. 4. Putative interpretation to explain the differential abundance in the SP + L vs SP comparison. The inclusion of lysozyme increases the availability of dietary protein to be digested by endogenous enzymes, by degrading the cell wall of Spirulina. This could lead to increased digestibility and consequent absorption of amino acids, which activates the mammalian target of rapamycin (mTOR) pathway, increasing the synthesis of muscle protein. Consequently, the needs for ATP in the muscle increase, which explains the higher abundance of TCA cycle enzymes. TPM3 - tropomyosin 3, TNNC1 – troponin C, MYL2/MYL3/MYL10 - myosin light chains, MDH2 – malate dehydrogenase, aconitate hydratase (ACO2). Created with BioRender.com.

daily gain of control pigs was significantly higher than that of Spirulina-fed pigs, regardless of enzyme inclusion [8].

4.2. Effect of Spirulina and lysozyme supplementation

Regarding the SP + L vs control comparison, only 3 proteins were detected with differential abundance. L-lactate dehydrogenase and thioredoxin were highly abundant in control, whereas apolipoprotein A-I was more abundant in the SP + L group. The latter, a major component of high-density lipoproteins (HDL), was reported by Wang et al. [37] to be positively related to adipose tissue deposition in the muscle tissue of indigenous Chinese pig breeds. Indeed, APOA1 participates in the PPAR signalling pathway, which promotes adipogenic differentiation [38]. Our previous results have shown that there are no significant differences in intramuscular fat content between SP + L and control piglets [8]. However, they had higher abundance of some PUFA, such as 18:3n-6 and 20:3n-6 compared to control. The higher abundance of APOA1, which also has antioxidative properties [39], could improve oxidative stability of this tissue as a reaction to PUFA enrichment. Finally, it is worth mentioning other roles in which this protein may be involved. Indeed, HDL [39] transports cholesterol from peripheral tissues towards the liver. There, it can be used for the synthesis of bile salts, which are actively secreted in the duodenum, to facilitate lipidic digestion. In the future, analysing the hepatic proteome of these piglets should unveil if APOA1 differential abundance found herein is coherent with hepatic cholesterol metabolism.

Conversely, the control group has higher abundance of LDH, a glycolytic enzyme that catalyses the conversion of lactate into pyruvate. This enzyme was reported to be highly abundant in the muscle of pigs fed with ractopamine, a supplement that promotes protein accretion and lipolysis, generating leaner meat [40]. In addition, Kwasiborsky et al. [41] reported a negative correlation between lightness (L*) and LDH in the muscle of outdoor reared pigs. Thus, the higher abundance of LDH in the control group could occur due to increased muscle development compared to SP + L, which is coherent with previous findings. Moreover, the higher abundance of LDH in control piglets could be related with meat colour, since these had the lowest L* value compared to piglets fed with diets with Spirulina inclusion [8].

4.3. Effect of lysozyme supplementation within Spirulina diets

Twelve proteins were detected with differential abundance in the SP + L vs SP comparison. The SP group had higher abundance of PYGM. This protein has already been mentioned due to its role in muscle development and glycogen metabolism. In this context, it seems that SP piglets are degrading collagen to a higher extent than SP + L. This seems to be corroborated by our results regarding feed digestibility: SP + L had higher DM digestibility than SP [8]. This could cause SP piglets to degrade endogenous nutrient reserves to a larger extent, which does not happen in the SP + L group due to the positive action of the lysozyme over nutrient digestibility.

Conversely, the SP + L group had higher abundance of several structural/contractile apparatus proteins: tropomyosin 3 (TPM3), troponin C (TNNC1), and several myosin light chains (MYL2, MYL3, MYL10). This pattern is again likely related to improved nutrient availability. Despite the fact that crude protein digestibility had no differences between SP and SP + L groups, dry matter digestibility was improved as a consequence of enzymatic supplementation [8]. Additionally, enhanced amino acid absorption rate, particularly of branch-chain amino acids (BCAA), could be causing enhanced muscle protein

synthesis. In fact, it has been reported that supplementing diets with 14% CP with isoleucine, leucine and valine (BCAA) temporarily prevents decreased growth performance of piglets during the first two-weeks after weaning [42]. This supplementation has also been reported to enhance the growth performance of piglets fed with 17% CP + BCAA to levels obtained with piglets fed with 20% CP [43]. Leucine inhibits protein degradation and promotes protein synthesis, likely to be via the mammalian target of rapamycin (mTOR) signalling pathway [44]. It has been suggested that simultaneous supplementation of all three BCAA promote muscle protein synthesis. Glutamine also promotes muscle protein synthesis, possibly through the mTOR signalling pathway [44]. Moreover, BCAA have a positive effect over intestinal development [45]. Coincidently, the SP + L group had the longest small intestine of all groups [8]. The results obtained in the present study could derive from the effect of lysozyme supplementation over amino acid digestibility. Thus, in future studies, it would be of paramount importance to evaluate individual amino acid digestibility at the ileal level, as well as plasma and tissue AA profiles, as performed before in small ruminants [46]. This would surely confirm or dismiss the aforementioned role of improved amino acid availability over muscle development based on high structural/contractile protein synthesis. Moreover, further research evaluating the impact of these dietary treatments over the gut microbiome/metaproteome would be of paramount importance. Indeed, diet is a major determinant of the gut microbiome composition [47], which has a major role on the health of the recently weaned piglet. This was recently demonstrated in piglets fed with seaweed extracts [48]. Combined with a metaproteomics approach [49] in order to assess the activity of these microorganisms, such study would contribute to determine the first impact of dietary treatments prior to tissue metabolism, providing a holistic view.

Finally, the SP + L group had higher abundance of malate dehydrogenase (MDH2) and aconitate hydratase (ACO2) compared to SP. Both of these are mitochondrial enzymes, involved in the TCA cycle, which releases stored energy via the oxidation of reserve carbohydrates, fat and/or proteins. Previous studies have indicated that the upregulation of mitochondrial TCA cycle is negatively correlated with feed efficiency in pigs, indicating that these generate lower levels of ATP in this organelle [50]. However, in the context of the present study, the higher abundance of MDH2 and ACO2 most likely derives from the increased abundance of contractile apparatus proteins, whose functions requires high input of ATP. Fig. 4 depicts the possible chain of events that explain this relationship.

4.4. Conclusions and future perspectives

In this study, the influence of dietary Spirulina on piglet muscle proteome was described for the first time. Additionally, the effect of dietary lysozyme supplementation was evaluated. Piglets fed with this microalga had altered muscle glycogen metabolism and endogenous nutrient mobilization, which could possibly be related with lower nutrient digestibility compared with others fed a diet without microalga. Interestingly, the SP + L group had higher abundance of structural and TCA cycle proteins compared to SP. Together with its beneficial effect over dry matter digestibility, it seems feasible that this differential abundance is influenced by increased availability of amino acids. Furthermore, our study also demonstrates the importance of combining novel omics approaches with other classical methods, such as tissue AA profiling.

In future studies, assessing AA digestibility, particularly at the ileal level, could validate the hypothesis of positive effect of lysozyme over AA digestibility. Also, the muscle AA profile would be helpful to assess the activity of the mTOR pathway. Additionally, the study of other tissue proteomes, such as liver and small intestine, will also be of relevance.

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Appendix A. Supplementary data

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