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Effect of dietary incorporation of *Chlorella vulgaris* and CAZyme supplementation on the hepatic proteome of finishing pigs

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ABSTRACT

The combination of several factors, including an increase in world population and living standards in developing countries and world dependency on conventional crop imports drive a search for alternative feedstuffs for poultry and pig diets. This would reduce the environmental impact associated with the foreseeable increase in the demand for animal products. One of such alternatives are microalgae, a diverse group of aquatic organisms with interesting nutritional properties. *Chlorella vulgaris* is a green microalga with a crude protein content comparable to that of soybean meal. However, its recalcitrant cell wall prevents it from being used as a nutrient source in monogastric diets. CAZyme supplementation is a putative strategy to increase its nutritional value, aiming at disrupting the cell wall and make intracellular nutrients available for digestion. The impact of these dietary strategies on the hepatic metabolism is currently unknown. The objective of this study was to evaluate the hepatic proteome of pigs fed with 5% *C. vulgaris* with or without CAZyme supplementation. Microalga inclusion has affected lipid metabolism and oxidative stress. CAZyme supplementation has caused higher oxidative stress in the liver, possibly caused by the higher digestive availability and consequent hepatic oxidation of fatty acids.

Significance: C. vulgaris, a microalga, is a novel feedstuff that is an alternative to conventional crops such as maize and soybean meal. Its recalcitrant cell wall may cause antinutritional effects when included in monogastric diets. This can be prevented by using exogenous enzyme supplementation, namely CAZymes, aimed at degrading this cell wall during digestion. Liver proteomics was used to identify the impact of these diets in finishing pig metabolism.

1. Introduction

The combined growth and increase in living standards of human populations is expected to significantly increase the demand for animal products such as pork or poultry, leading the industry to increase its production whilst having to maintain the environmental sustainability of the production chains [1]. Indeed, monogastric feed industry, and particularly for pig production, is heavily dependent on conventional crops, namely cereals and soybean meal (SBM), the latter as a protein source. The production of these crops requires the extensive use of natural resources and has been considered a contributing factor to deforestation in South America [2]. Additionally, Europe is heavily dependent on SBM imports, and consequent greenhouse gas emissions and high transport costs, which worsens the environmental impact and

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costs of pig production [3]. Therefore, the search for alternative, sustainable and viable feedstuffs, within the context of a circular economy, became an important topic in recent years, with the scientific community focusing on the use of agri-food chain by-products [4,5], such as insect meals [6,7] or algae [8,9]. The latter comprise a vast array of organisms from unicellular microalgae, such as *C. vulgaris*, to multicellular seaweeds, such as *Ulva lactuca*.

Microalgae include eukaryotic diatoms (*Bacillariophyceae*), green algae (*Chlorophyceae*) and golden algae (*Chrysophyceae*) [10]. These diverse groups have high potential for being used in livestock diets, due to their crude protein contents and bioactive compounds, such as pigments and n-3 PUFA. In particular, *C. vulgaris* has a CP content similar to that of SBM (up to 48% in dry matter [10]). However, most studies report inclusion levels that resemble supplements/additive levels as opposed to nutrient sources [11]. Such studies determined for instance that supplement levels of *C. vulgaris* (0.1–0.2%) increases the average daily gain (ADG) of growing pigs whilst lower levels (0.0002%) have no consequences on either performance or meat quality [10]. The structural complexity of their cell wall carbohydrates inhibits microalgae utilization as a nutrient source in monogastric diets, where high percentages of inclusion can have anti-nutritional effects [8].

In order to improve their nutritional value, external carbohydrate-active enzyme (CAZyme) supplementation could be used to degrade the cell wall and allow the animal's endogenous enzymes to act upon the feed during digestion [12]. Indeed, this process has been previously reported in weaned piglets fed with another microalga, Spirulina (*Arthrospira platensis*), allowing an increase in dry matter digestibility compared to a Spirulina-fed group without enzyme [8]. However, enzyme supplementation did not improve animal performance to the levels of control. In finishing pigs, this problem is not as severe due to mature gastrointestinal tract of these animals. However, CAZyme inclusion enables better utilization of the micronutrients from the microalgae, which in turn enhances meat quality traits and nutritional value. Indeed, finishing pigs fed with 5% *C. vulgaris* had lower saturated fatty acids (SFA) and n-6:n-3 ratio in their meat compared to control [13]. In addition, supplementing the microalgae diet with a mix of CAZymes [12] increased the DPA (22:5 n-3) and DHA (22:6 n-3) content of meat by 1.6-fold compared to control [13]. This was accomplished without any detrimental effects in growth performance. Hence, *C. vulgaris* was found to be a viable way to replace soybean meal whilst improving the quality of pork. However, the underlying consequences of microalgae and CAZyme supplementation in the metabolism of pigs are not fully understood. To fully explore them, Omics - particularly proteomics, are interesting tools to explore tissue metabolism as affected by diet [14].

The advent of proteomics, particularly shotgun/label-free proteomics, has brought immense possibilities to the field of animal science. Most interestingly, it allows an in-depth perspective on the molecular repercussions of a study factor upon a tissue, organ, fluid, organism or population. This technology has enabled for instance the establishment of putative biomarkers for seasonal weight loss tolerance [15] or the study of increased intramuscular fat in pigs [16], among many other subjects. In the context of pork production, it can be used to find biomarkers that distinguish different breeds and muscle types [17], which ultimately improves decision making and traceability based on this data [18,19]. Most proteomic research in pigs as a production species focuses on the muscle, which is the tissue of higher economic value [19]. As the liver is the centre of major metabolic pathways in the mammalian organism, it justifies studying it to assess metabolic impact in the context of pig production. Cui et al. [20] for instance studied the impact of heat stress in the hepatic proteome of finishing pigs. In another example, Bovo et al. [21] studied the hepatic proteome to differentiate metabolic signatures of two different pig breeds. However, to our knowledge, there is no information regarding the impact of dietary microalgae in the hepatic proteome of pigs. The objective of this study was to evaluate the hepatic proteome of finishing pigs as affected by dietary *C. vulgaris* and exogenous CAZymes and assess the metabolic and physiological implications of such use.

2. Materials and methods

2.1. Live animal trial and experimental conditions

The procedures in the live animal trial have been previously described [13] and are mentioned here for contextualization purposes. Briefly, forty entire male pigs [(Large White × Landrace) female × Pietrain males] with an initial live weight of 59.1 ± 5.69 kg were distributed into 10 pens with 4 animals each, equipped with individual creep feeders that allow control of individual intake in each pen. Each animal was individually fed with one of four experimental diets ($n = 10$): cereal and SBM-based diet (control), MA – control diet with 5% *C. vulgaris* (Allmicroalgae, Pataias, Portugal), MAR – MA with 0.005% Rovabio Excel AP (Adisseo, Antony, France) and MAM – MA with 0.01% four CAZyme mixture (*exo*-β-glucosaminidase, alginate lyase, peptidoglycan *N*-acetylmuramic acid deacetylase and lysozyme) as described by Coelho et al. [12]. Rovabio Excel AP is a mix of commercially available enzymes that include xylanases, β-glucanases, cellulases, pectinases and proteases. The ingredients and proximal composition of experimental diets are presented in Supplementary Table 1. The pigs were fed *ad libitum* and had free access to water. Upon reaching a body weight of 101 ± 1.9 kg, animals were slaughtered with electrical stunning followed by exsanguination following standard procedures used in commercial abattoirs in accordance with Portuguese and EU legislation. As described, the experiment was approved by the Animal Care Committee of the National Veterinary Authority (Direção-Geral de Alimentação e Veterinária), following the appropriated European Union guidelines (2010/63/EU Directive). Upon slaughter, the whole liver was collected, and samples excised with a scalpel blade, snap-frozen in liquid Nitrogen and kept at −80 °C until further analysis.

2.2. Protein extraction and sample preparation

The methodology used for proteomic analysis was based on a previously published protocol [22]. Briefly, six samples were randomly chosen per experimental group ($n = 6$). They were processed in tubes containing lysing matrix A (MP Biomedicals, Irvine, CA, USA) and lysis buffer (100 mM Tris-HCl pH 8.5, 1% sodium deoxycholate (SDC), 10 mM tris (2-carboxyethyl) phosphine (TCEP), 40 mM chloroacetamide (CAA) and protease inhibitors. Protein homogenization was performed using the FastPrep-24 equipment (MP Biomedicals, Eschwege, Germany) at 6 m/s in 3 cycles of 30 s each, with intervals of 5 min in ice. Protein extracts were then centrifuged for 5 min at 13,400 rpm and transferred into 1.5 mL low protein binding tubes. All extracts were incubated for 10 min at 95 °C under agitation (Thermomixer, Eppendorf, Hamburg, Germany), then sonicated for ten cycles of 30 s at 4 °C (Bioruptor, Diagenode, Liège, Belgium) and centrifuged again. The lysate was transferred onto a new 1.5 mL tube. Then, 100 μg of protein from each sample was processed for proteomic analysis. Enzymatic digestion was performed with trypsin/LysC (2 micrograms) overnight at 37 °C at 1000 rpm. Peptide concentration was measured by fluorescence.

2.3. Label-free analysis and mass spectrometry

Protein identification and quantitation were performed by nanoLC-MS/MS using an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). Five hundred nanograms of peptides of each sample were loaded onto a trapping cartridge (Acclaim PepMap C18 100 Å, 5 mm × 300 μm i.d., 160,454, Thermo Scientific, Bremen, Germany) in a mobile phase of 2% ACN, 0.1% FA at 10 μL/min. After 3 min loading, the trap column was switched in-line to a 50 cm × 75 μm inner diameter EASYSpray column (ES803, PepMap RSLC, C18, 2

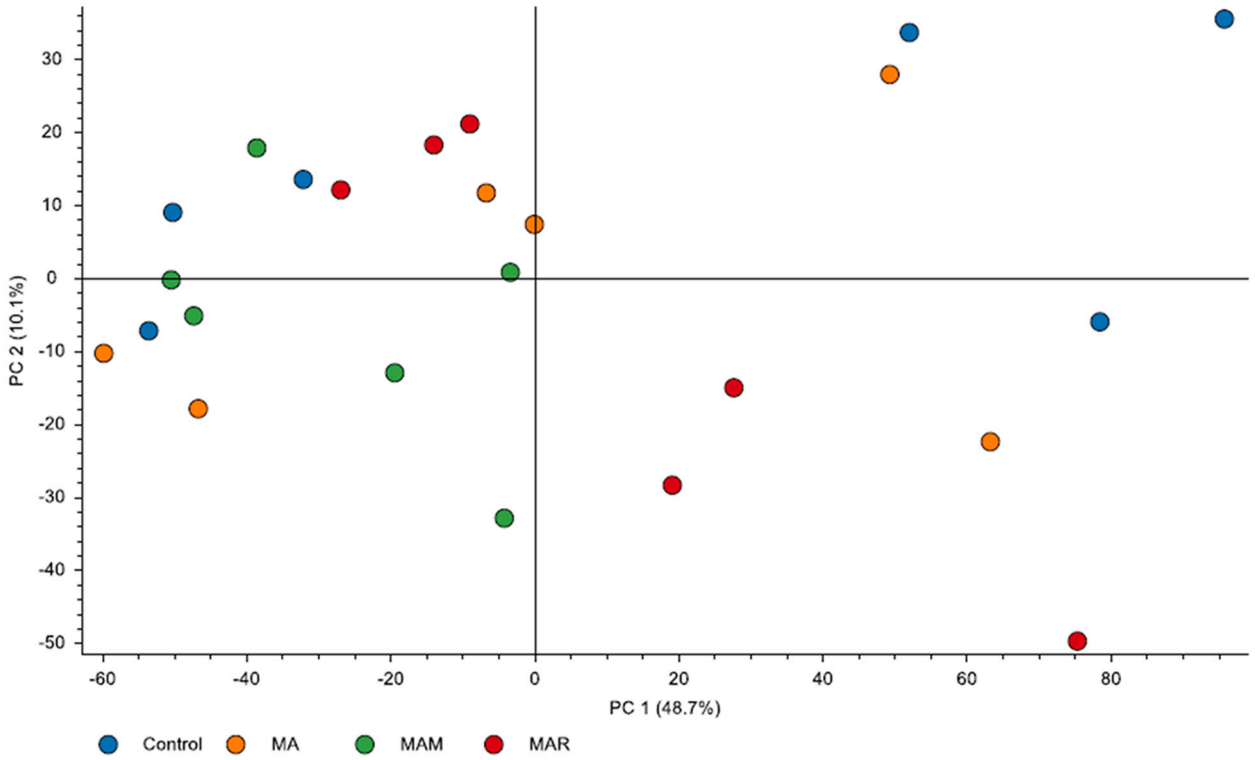

Fig. 1. Principal component analysis of proteins identified in the liver of pigs fed with control, MA (5% *Chlorella vulgaris*), MAR (5% *C. vulgaris* + 0.005% Rovabio Excel AP) and MAM (5% *C. vulgaris* + 0.01% of a four-CAZyme mixture) diets.

μm, Thermo Scientific, Bremen, Germany) at 250 nL/min. Separation was achieved by mixing A: 0.1% FA and B: 80% ACN, 0.1% FA with the following gradient: 5 min (2.5% B to 10% B), 120 min (10% B to 30% B), 20 min (30% B to 50% B), 5 min (50% B to 99% B), and 10 min (hold 99% B). The column was equilibrated with 2.5% B for 17 min. Data acquisition was controlled by Xcalibur 4.0 and Tune 2.9 software (Thermo Scientific, Bremen, Germany).

The mass spectrometer was operated in the data-dependent (dd) positive acquisition mode alternating between a full scan (*m/z* 380–1580) and subsequent HCD MS/MS of the 10 most intense peaks from a full scan (normalized collision energy of 27%). The ESI spray voltage was 1.9 kV. The global settings were as follows: use lock masses best (*m/z* 445.12003), lock mass injection Full MS and chromatographic peak width (FWHM) of 15 s. The full scan settings were as follows: 70 k resolution (*m/z* 200), AGC target 3×10^6, maximum injection time 120 ms; dd settings: minimum AGC target 8×10^3, intensity threshold 7.3×10^4, charge exclusion: unassigned, 1, 8, >8, peptide match preferred, exclude isotopes on, and dynamic exclusion 45 s. The MS2 settings were as follows: microscans 1, resolution 35 k (*m/z* 200), AGC target 2×10^5, maximum injection time 110 ms, isolation window 2.0 m/z, isolation offset 0.0 m/z, dynamic first mass, and spectrum data type profile.

2.4. Data treatment and analysis

The raw data were processed using the Proteome Discoverer 2.4.0.305 software (Thermo Scientific, Bremen, Germany). Protein identification analysis was performed with the data available in the UniProt protein sequence database for the *Sus scrofa* Proteome (2019_11) with 49,571 entries. The Sequest search node was considered with an ion mass tolerance of 10 ppm and 0.02 Da for precursor and fragment ions, respectively. Missed cleavage tolerance was set to 2. Cysteine carbamidomethylation was defined as a fixed modification. The following variable modifications were considered: methionine oxidation, protein N-terminus acetylation, loss of methionine and Met-loss+Acetyl. Peptide confidence was set to high. The processing node Percolator was enabled with the following settings: maximum delta Cn 0.05; decoy database search target False Discovery Rate—FDR 1%; validation based on q-value. Protein-label-free quantitation was performed with the Minora feature detector node at the processing step. Precursor ion quantification was performing at the processing step with the following parameters: Peptides: unique plus razor; precursor abundance was based on intensity; normalization mode was based on the total peptide amount; the hypothesis test was based on a *t*-test (background based).

For determination of differentially abundant proteins between experimental conditions, the following filters were considered following previously published protocols [23,24]: the number of unique peptides was set to 2 (minimum), the *p*-value set to <0.05 and each protein had to be identified in at least half the samples. The protein fold change, calculated as (X-Y)/Y, between comparisons was set to >2 (high abundance) and < −0.67 (low abundance). A Principal Component Analysis (PCA) of identified proteins was carried out using R software. A heatmap of differentially abundant proteins was carried out using the ClustVis platform [25]. Differentially abundant proteins were classified according to molecular function (MF), biological process (BP) and cellular component (CC) using the PANTHER classification system [26]. Functional enrichment analysis was carried out following the protocol described by Sirri et al. [27], using the ClueGO [28] app of the Cytoscape [29] software.

The MS proteomics data has been uploaded to the ProteomeXchange Consortium [30] via the PRIDE [31] partner repository with the dataset identifier PXD028361.

3. Results

3.1. Animal performance and carcass characteristics

Diet had no influence on any parameter concerning growth

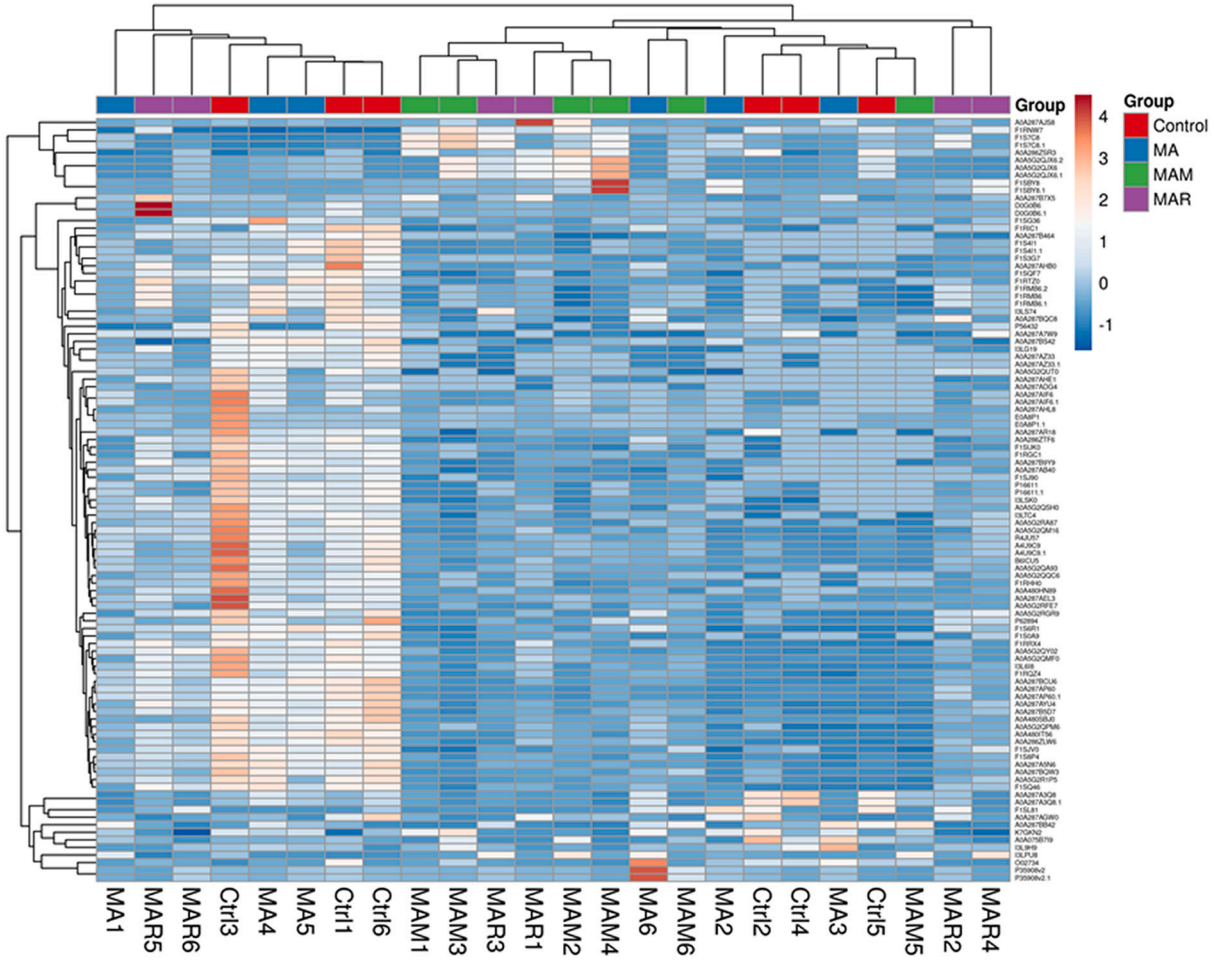

Fig. 2. Heatmap of differentially abundant proteins in the hepatic proteome of finishing pigs fed with control, MA (5% *Chlorella vulgaris*), MAR (5% *C. vulgaris* + 0.005% Rovabio Excel AP) and MAM (5% *C. vulgaris* + 0.01% of a four-CAZyme mixture) diets.

performance, including final weight, average daily gain and feed conversion ratio. Considering carcass characteristics, only perirenal fat had statistically significant differences, which was lower in MA compared to control animals. For a more detailed description of results, kindly refer to the companion paper by Coelho and co-workers [13].

3.2. Hepatic proteome

A total of 4663 proteins were identified in the hepatic proteome of these pigs, 4337 of which with a high protein FDR confidence (Supplementary material 1). The PCA carried out (Fig. 1) shows that the experimental groups do not cluster considering the first two principal components.

3.3. Differential protein abundance

A total of 97 proteins were detected with differential abundance. A Venn diagram is provided in supplementary material 2, depicting common proteins between comparisons. The heatmap obtained for these proteins of all comparisons (Fig. 2) had no major clustering of any group. Regarding the GO classification (Fig. 3), most proteins (51.50%) had binding as the major molecular function (MF), followed by catalytic activity (27.30%). The biological process (BP) category found more classes, the most abundant being cellular process (34.60%) and metabolic process (25.60%). The cell component (CC) category returned three cellular locations: cellular anatomical entity (45.90%), intracellular (37.70%) and protein-containing complex (16.40%). Functional enrichment analysis yielded an interaction network only for the MAM vs control comparison, as seen in Fig. 4, with represented proteins belonging only to the control group. These were related to biological process annotations only, namely mitotic nuclear membrane reassembly (GO:0007084), positive regulation of DNA binding (GO:0043388) and phosphatase regulator activity (GO:0019208).

The MA vs control comparison had 2 differentially abundant proteins (Table 1). Both were more abundant in the control group: Transcription factor AP-1 (JUN) and Insulin-like growth factor-binding protein 1 (IGFBP1) with DNA and IGF binding molecular functions, respectively.

The MAR vs control comparison yielded 12 proteins with differential abundance (Table 2), four of them, over-abundant in the MAR group: eukaryotic translation initiation factor 4 gamma 3 (EIF4G3) that binds mRNA, Carnitine O-octanoyltransferase (CROT), Heterogeneous nuclear ribonucleoprotein L (HNRNPL) and Perilipin (ADFP) that participates in lipid storage.

The MAM vs control comparison had 62 differentially abundant proteins (Supplementary Table 2). Only three of them were highly abundant in the MAM group: an uncharacterized protein (LOC100515185), Glutathione S-transferase alpha 4 (GSTA4) and Carn_acyltransf domain-containing protein (CROT), the latter two participating in glutathione and coenzyme A metabolic process, respectively.

The MAR vs MA comparison had nine differentially abundant proteins (Table 3), only three of them being higher in the MAR group, such as Perilipin (ADFP) whose role in lipid storage has already been mentioned. The remaining proteins that are highly abundant in MA include Hydroxymethylglutaryl-CoA synthase (HMGCS2).

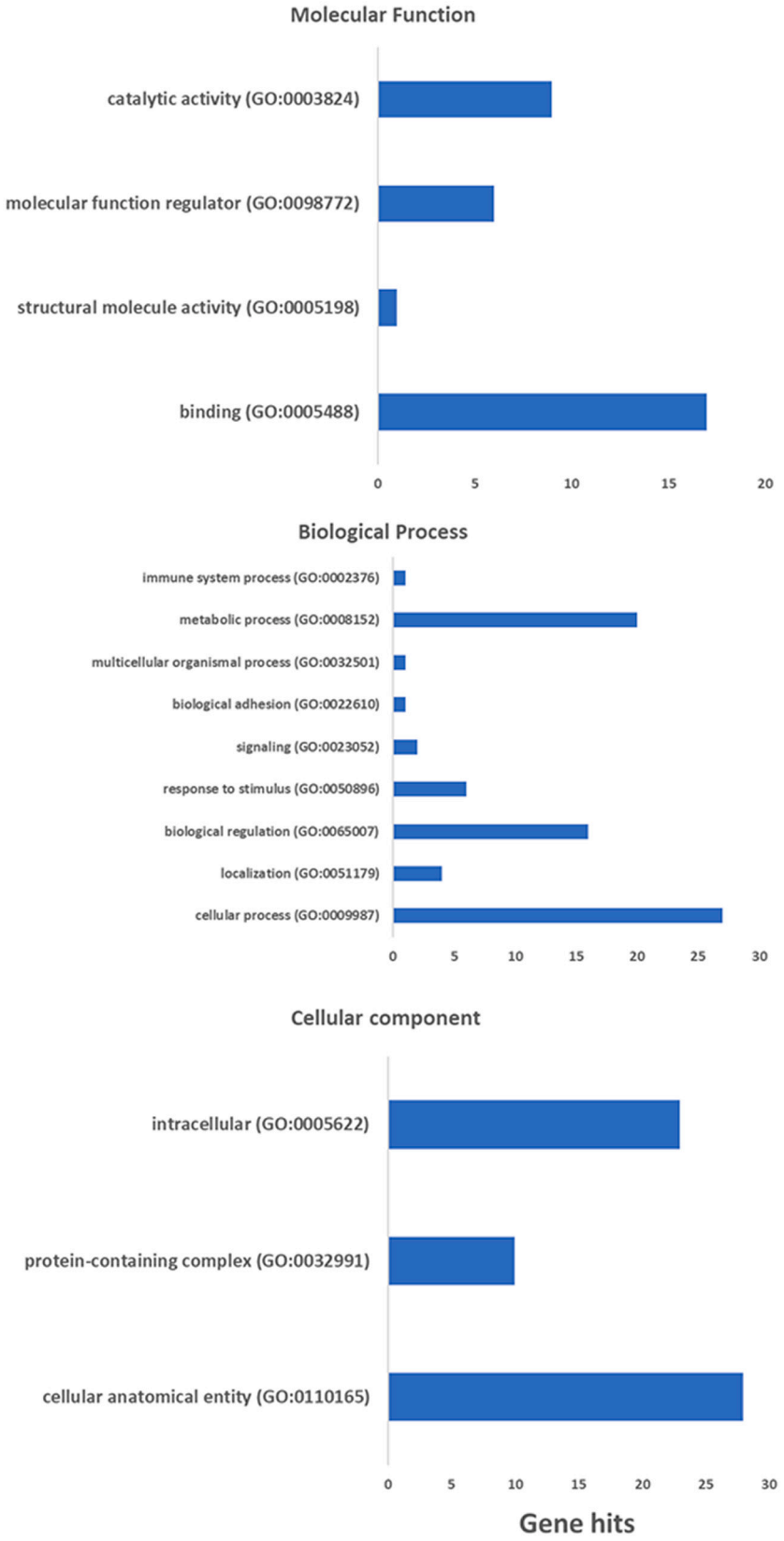

Fig. 3. Gene ontology classification of differentially abundant proteins.

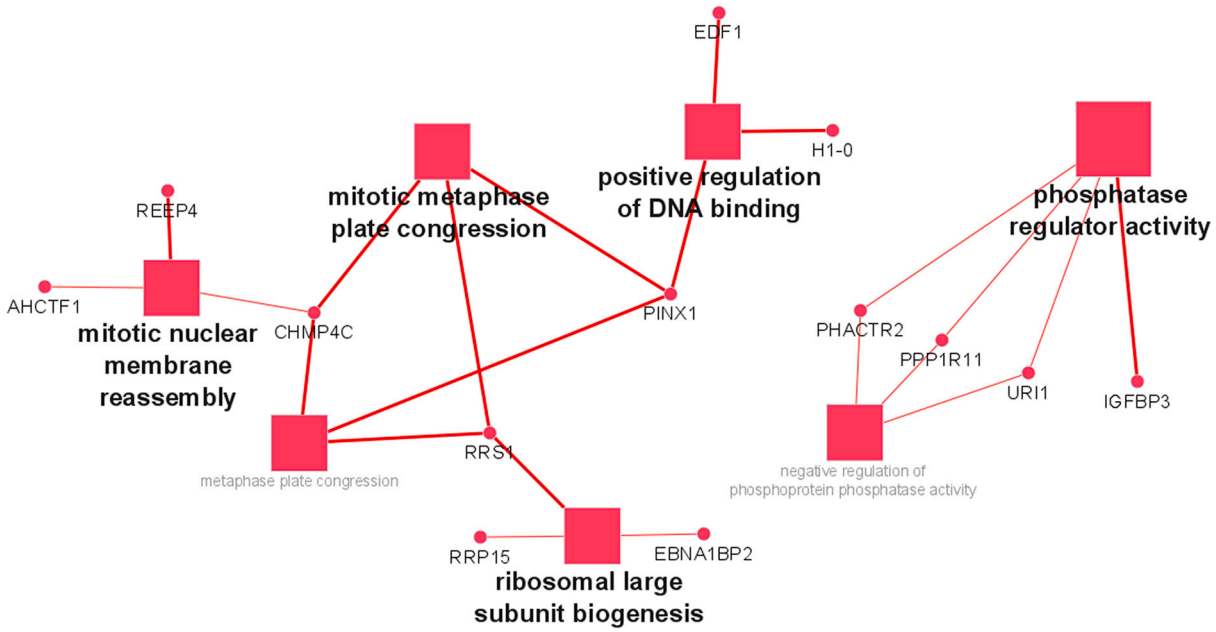

Fig. 4. Functional network analysis obtained for differentially abundant proteins of the MAM vs control comparison. Squares represent biological process annotations and circles are proteins (gene IDs) highly abundant in the control group.

Table 1
Differential protein abundance in MA (5% *Chlorella vulgaris*) vs control.

Protein accession	Protein description	Gene symboL	Fold changE	*P* value	Unique peptides
P56432	Transcription factor AP-1	NHSL1	−0.77	0.003	2
E0A8P1	Insulin-like growth factor-binding protein 1	CA3	−0.75	0.014	2

The MAM vs MA comparison yielded 11 proteins with differential abundance (Table 4), such as acyl-coenzyme A oxidase (ACOX1) and glutathione S-transferase alpha 4 (GSTA4), who were highly abundant in the MAM group. The proteins are involved in PUFA oxidation and antioxidant metabolism, respectively.

The MAM vs MAR comparison had one protein identified with differential abundance in this comparison (Table 5), with higher abundance in MAR group: receptor expression enhancing protein (REEP4).

4. Discussion

Omics applications in nutrition-oriented studies have been extensively described. However, using proteomics to study the impact of dietary microalgae and CAZyme supplementation on pig liver metabolism has been – to our knowledge – absent from literature until this point. As demonstrated in the PCA plot previously mentioned (Fig. 1), there was not a clear clustering of identified proteins throughout the different experimental groups. Overall, this means that the experimental dietary treatments had an impact on pig metabolism similar to that of the control diet, which lead to a lack of significantly altered pathways between experimental groups. This has been the case in previous studies by our research team, where dietary treatments led to changes mostly concerning individual proteins [32–34]. Nevertheless, the experimental design led to changes at the hepatic proteome level that warrant further clarification and provide interesting clues to the metabolic effects of the use of both *C. vulgaris* and CAZymes use. In this section we have highlighted the most relevant relationships found.

Table 2
Differential protein abundance in MAR (5% *Chlorella vulgaris* +0.005% Rovabio Excel AP) vs control.

Protein accession	Protein description	Gene symbol	Fold change	P value	Unique peptides
E0A8P1	Insulin-like growth factor-binding protein 1	IGFBP1	−0.88	<0.001	2
A0A075B7I9	Ig-like domain-containing protein	–	−0.82	<0.001	2
A0A287A3Q8	CAP-Gly domain containing linker protein 2	CLIP2	−0.80	<0.001	2
A0A5G2RFE7	Prothymosin alpha	PTMA	−0.80	0.002	3
F1S4I1	Golgi membrane protein 1	GOLM1	−0.76	0.019	4
A0A287AIF6	Death inducer-obliterator 1	DIDO1	−0.75	0.010	5
P16611	Insulin-like growth factor-binding protein 3	IGFBP3	−0.73	0.017	5
F1RGC1	Suppression of tumorigenicity 5	ST5	−0.68	0.056	4
D0G0B6	Perilipin	ADFP	2.01	0.054	15
I3LPU8	Heterogeneous nuclear ribonucleoprotein L	HNRNPL	2.59	0.007	3
A0A287B7X5	Eukaryotic translation initiation factor 4 gamma 3	EIF4G3	3.17	0.001	2
F1SBY8	Carn_acyltransf domain-containing protein	CROT	3.34	<0.001	18

4.1. Comparison of MA vs control groups

This comparison yielded two highly abundant proteins in control. Indeed, these pigs had a higher abundance of IGFBP1, a protein that binds to IGF-1 and regulates its activity. Dietary factors have been reported to influence IGF-1 activity in pigs [35]. Due to the structural similarity to insulin, IGF-1 can bind to insulin receptors [36]. By

Table 3
Differential protein abundance in MAR (5% *C. vulgaris* +0.005% Rovabio Excel AP) vs MA (5% *C. vulgaris*).

Protein accession	Protein description	Gene symbol	Fold change	P value	Unique peptides
A0A287BB42	Estradiol 17-beta-dehydrogenase 2	HSD17B2	−0.77	<0.001	11
I3L9H9	Uncharacterized protein	CYP2J34	−0.76	<0.001	4
O02734	Hydroxymethylglutaryl-CoA synthase, mitochondrial	HMGCS2	−0.73	0.004	12
A0A287BS42	Fermitin family member 3	FERMT3	−0.72	0.058	5
K7GKN2	Interferon-induced GTP-binding protein Mx1	MX1	−0.70	0.008	7
A0A287AIF6	Death inducer-obliterator 1	DIDO1	−0.67	0.028	5
A0A5G2QJX6	Uncharacterized protein	LOC100515185	2.17	<0.001	9
A0A287AJS8	Ig-like domain-containing protein	SLA-2	2.78	<0.001	2
D0G0B6	Perilipin	ADFP	3.84	0.040	15

Table 4
Differential protein abundance in MAM (5% *C. vulgaris* +0.01% of a four-CAZyme mixture) vs MA (5% *C. vulgaris*).

Protein accession	Protein description	Gene symbol	Fold change	P value	Unique peptides
A0A287AZ33	Uncharacterized protein	WIZ	−0.90	<0.001	2
A0A480SBJ0	CCCTC-binding factor	CTCF	−0.80	0.048	3
F1RMB6	Receptor expression-enhancing protein	REEP4	−0.79	0.008	2
A0A287AP60	DnaJ heat shock protein family (Hsp40) member B14	DNAJB14	−0.77	0.010	2
F1RIC1	Coiled-coil domain-containing protein 86	CCDC86	−0.69	0.041	2
F1S6R1	Cold-inducible RNA-binding protein isoform 1	DAZAP1	−0.69	0.010	4
A0A287AHE1	Testis development related protein	TDRP	−0.68	0.010	2
F1S7C8	Glutathione S-transferase alpha 4	GSTA4	2.53	0.001	2
F1RNW7	G-patch domain containing 1	GPATCH1	2.65	0.011	3
A0A286ZSR3	Acyl-coenzyme A oxidase	ACOX1	2.99	<0.001	2
A0A5G2QJX6	Uncharacterized protein	LOC100515185	6.40	<0.001	9

Table 5
Differential protein abundance in MAM (5% *C. vulgaris* +0.01% of a four-CAZyme mixture) vs MAR (5% *C. vulgaris* +0.005% Rovabio Excel AP).

Protein accession	Protein description	Gene symbol	Fold change	P value	Unique peptides
F1RMB6	Receptor expression-enhancing protein	REEP4	−0.78	0.016	2

increasing IGFBP1 abundance, control pigs may putatively increase fatty acid mobilization in comparison to MA, through inhibition of IGF-1 activity.

4.2. Comparison of MAR vs control groups

A total of 12 proteins were differentially abundant in this comparison, four of them in MAR. Perilipin (ADFP), a protein that takes part in lipid storage and long-chain fatty acid transport, is highly abundant in MAR by comparison to the control animals. This protein promotes the formation and stabilization of lipid droplets and is involved in the storage of lipid depots [43]. These pigs have been reported of accumulating higher levels of PUFA in the muscle, such as 18:3 n-3, than those from control [13]. Therefore, it could be suggested that PUFA accumulation is occurring in the liver because of dietary *C. vulgaris* intake. However, they also had higher abundance of CROT, a protein involved in the β-oxidation of fatty acids in peroxisomes. In human liver cells, the overexpression of CROT is related with lower PUFA contents [37]. CROT abundance could putatively result from increased availability of PUFA for oxidation.

Moreover, control pigs had higher abundance of IGFBP1 and IGFBP3 in comparison to MAR, similarly to what was mentioned in the first comparison. These have been reported to regulate cell development in pigs [38], in addition to influence energy metabolism.

4.3. Comparison of MAM vs Control groups

This comparison yielded the highest number of differentially abundant proteins, with only three of them being highly abundant in MAM vs control: an uncharacterized protein, CROT and glutathione S-transferase alpha 4 (GSTA4). As mentioned earlier, CROT is a protein involved in the β-oxidation of fatty acids, a process that generates reactive oxygen species (ROS) such as H_2O_2 [39]. Interestingly, these same pigs had a higher abundance of GSTA4, a protein that participates in glutathione metabolism, which is of paramount importance for tissue detoxification from ROS, for example. Ukropec et al. [40] have found that feeding rats with high-fat diets containing PUFA increases liver fatty acid oxidation. The same could be happening in these pigs, since theoretically MAM pigs have higher dietary availability of PUFA than control pigs. The increased abundance of GSTA4 is a putative response to enhance tissue ROS detoxification.

The remaining proteins were all more abundant in control pigs. In line with the two previous comparisons, control pigs had higher abundance of IGFBP3. They also had higher abundance of collagen (COL5A3), a basilar component of the extracellular matrix, while simultaneously having higher abundance of proteins that participate in protein ubiquitination (RCR-type E3 ubiquitin transferase - MYCBP2) and ubiquitin-dependent protein catabolism (E3 ubiquitin-protein ligase - PPP1R11). The reason why they had these seemingly contradictory results is unclear, but it could derive from higher protein turnover rates in control pigs compared to MAM. Indeed, our functional analysis supports this hypothesis, since this group had higher abundance of RRP15-like protein (RRP15) and EBNA1 binding protein 2 (EBNA1BP2), two proteins related to ribosomal large subunit biogenesis, a process required for ribosomal assembly and ultimately, protein synthesis.

4.4. Comparison of MAR vs MA groups

Nine proteins were found with differential abundance in this comparison, with seven of them being highly abundant in MA, such as HMGCS2. This protein participates in ketogenesis and has been reported to be upregulated in the liver of diabetic pig models [41]. The reason why HMGCS2 is highly abundant in MA pigs is uncertain, but it could derive from higher fatty acid oxidation rates, and the concomitant availability of acetyl-CoA. Indeed, this could be the case since MAR pigs have putatively higher lipid storage in the liver, as indicated by their

higher abundance of perilipin (ADFP).

4.5. Comparison of MAM vs MA groups

In this comparison, 11 proteins were identified with differential abundance. Interestingly, MAM pigs had higher abundance of GSTA4, a transferase that catalyses aldehyde bond formation with glutathione for tissue detoxification. Acyl-coenzyme A oxidase (ACOX1), highly abundant in MAM, participates in the β-oxidation of fatty acids, which generates ROS such as hydrogen peroxide, increasing oxidative stress. Taken together, it could be suggested that MAM pigs are under higher levels of hepatic oxidative stress than those fed without the CAZyme mix. Indeed, we have found that pigs fed with *C. vulgaris* accumulate higher levels of n-3 PUFA in the muscle than those fed a control diet [13], albeit not negatively impacting tissue oxidative stability. Feeding rats with n-3 PUFA has been reported as a cause for increased hepatic fatty acid oxidation [40]. The effect of the CAZyme mix might be causing a higher metabolization of n-3 PUFA in the liver of MAM vs MA pigs, by increasing PUFA digestibility and consequently its hepatic metabolization. In the future, quantifying FA in the liver of pigs fed with MAM and MA diets would allow the confirmation of this hypothesis.

4.6. Comparison of MAM vs MAR groups

This comparison has resulted in the differential abundance of one protein - REEP4, with microtubule binding functions. The reason why the dietary treatments cause this difference is not clear. It could result from the fact that the diets were not stressing enough to cause significant differences in the hepatic proteome/metabolism, reflecting only differences in cellular organization due to individual variability.

4.7. Conclusions and future perspectives

This study evaluated, for the first time, the impact of dietary incorporation of *C. vulgaris* and enzyme supplementation on hepatic proteome of finishing pigs. We found that these dietary factors had no major effect on hepatic metabolism, albeit influencing individual proteins of the lipid metabolism and oxidative stress. Indeed, increasing the hepatic input of n-3 PUFA is likely behind the higher susceptibility of MAM to oxidative stress compared to MA. In turn, MAM is less susceptible to oxidative stress compared to control, eventually due to the combined effect of dietary *C. vulgaris* and CAZyme that putatively increase liver antioxidant content (e.g. carotenoids). As suggested in other previous studies [32,42], the use of classical biochemical analyses to study hepatic fatty acid and carotenoid profiles of these pigs would be necessary to confirm the hypothesis established above. Moreover, the analysis of muscle proteome would also be important to verify the impact of n-3 PUFA enrichment in this tissue's metabolism. Finally, studying the intestinal proteome would allow further insights into the impact of these dietary treatments on digestive physiology.

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