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Metabolomics and proteomics insights into hepatic responses of weaned piglets to dietary Spirulina inclusion and lysozyme supplementation

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Abstract

Background Studying the effect of dietary Spirulina and lysozyme supplementation on the metabolome and proteome of liver tissue contributes to understanding potential hepatic adaptations of piglets to these novel diets. This study aimed to understand the influence of including 10% Spirulina on the metabolome and proteome of piglet liver tissue. Three groups of 10 post-weaned piglets, housed in pairs, were fed for 28 days with one of three experimental diets: a cereal and soybean meal-based diet (Control), a base diet with 10% Spirulina (SP), and an SP diet supplemented with 0.01% lysozyme (SP + L). At the end of the trial, animals were sacrificed and liver tissue was collected. Metabolomics analysis ($n = 10$) was performed using NMR data analysed with PCA and PLS-DA. Proteomics analysis ($n = 5$) was conducted using a filter aided sample preparation (FASP) protocol and Tandem Mass Tag (TMT)-based quantitative approach with an Orbitrap mass spectrometer.

Results Growth performance showed an average daily gain reduction of 9.5% and a feed conversion ratio increase of 10.6% in groups fed Spirulina compared to the control group. Metabolomic analysis revealed no significant differences among the groups and identified 60 metabolites in the liver tissue. Proteomics analysis identified 2,560 proteins, with 132, 11, and 52 differentially expressed in the Control *vs.* SP, Control *vs.* SP + L and SP *vs.* SP + L comparisons, respectively. This study demonstrated that Spirulina enhances liver energy conversion efficiency, detoxification and cellular secretion. It improves hepatic metabolic efficiency through alterations in fatty acid oxidation (e.g., upregulation of enzymes like fatty acid synthase and increased acetyl-CoA levels), carbohydrate catabolism (e.g., increased glucose and glucose-6-phosphate), pyruvate metabolism (e.g., higher levels of pyruvate and phosphoenolpyruvate carboxykinase), and cellular defence mechanisms (e.g., upregulation of glutathione and metallothionein). Lysozyme supplementation mitigates some adverse effects of Spirulina, bringing physiological responses closer to control levels. This includes fewer differentially expressed proteins and improved dry matter, organic matter and energy digestibility.

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Lysozyme also enhances coenzyme availability, skeletal myofibril assembly, actin-mediated cell contraction, tissue regeneration and development through mesenchymal migration and nucleic acid synthesis pathways.

Conclusions While Spirulina inclusion had some adverse effects on growth performance, it also enhanced hepatic metabolic efficiency by improving fatty acid oxidation, carbohydrate catabolism and cellular defence mechanisms. The addition of lysozyme further improved these benefits by reducing some of the negative impacts on growth and enhancing nutrient digestibility, tissue regeneration, and overall metabolic balance. Together, Spirulina and lysozyme demonstrate potential as functional dietary components, but further optimization is needed to fully realize their benefits without compromising growth performance.

Keywords Piglets, Spirulina, Carbohydrase, Lysozyme, Liver proteome, Liver metabolome

Background

Pig production is a vital contributor to global food security, providing a substantial portion of the world's meat supply. As the demand for animal protein continues to rise, there is an increasing need to explore innovative strategies that enhance productivity while maintaining sustainability. Ensuring efficient use of feed resources, improving growth performance and promoting animal health are critical factors in meeting these demands. In this context, exploring alternative feed ingredients, such as microalgae, offers promising potential to improve nutritional value, reduce environmental impact, and enhance productivity. This study investigates the effects of dietary Spirulina inclusion and lysozyme supplementation on the hepatic metabolome and proteome of piglets, aiming to understand how these novel feed additives influence liver function and overall health. These commodities are usually imported from outside the European Union, raising concerns about economic and environmental sustainability related to their international trade [1]. Consequently, there is a pressing need to find full or partial replacements for these feedstuffs. The use of whole microalgae in animal diets has been explored as an alternative, mainly as a supplement [2–5] and, more recently, as a primary ingredient [6–15]. Microalgae's nutritional composition varies significantly between species, growth conditions and biomass status, but they are generally rich in proteins, lipids and carbohydrates, making them comparable to conventional feedstuffs [16].

Spirulina (*Arthrospira platensis*) is one of the most studied microalgae in animal feeding contexts, noted for its high crude protein (60–70%), crude fat (2–7%) and carbohydrate (18–23%) contents on a dry weight basis [17]. Additionally, Spirulina is enriched with n-3 long-chain polyunsaturated fatty acids (LC-PUFA), vitamins, minerals, carotenoids and other pigments, as well as various bioactive compounds, positioning it as a promising natural resource with significant health benefits for both animals and humans [18]. However, microalgae possess recalcitrant cell walls that complicate their digestion in monogastric animals, highlighting the need for technologies that enhance nutrient utilization and allow for the cost-effective inclusion of microalgae in animal diets [19, 20]. Carbohydrate-active enzymes (CAZymes) have been studied in vitro for their capacity to break down microalgae cell walls, thereby improving their nutritional value for monogastric animals and permitting higher dietary inclusion levels. For instance, Coelho et al. [21] demonstrated the effectiveness of a novel mixture of four CAZymes in disrupting the cell walls of *Chlorella vulgaris*. The use of whole microalgae in animal diets has been explored as an alternative, mainly as a supplement and more recently as a primary ingredient. Microalgae's nutritional composition varies significantly between species, growth conditions and biomass status, but they are generally rich in proteins, lipids and carbohydrates, making them comparable to conventional feedstuffs. However, understanding how these alternative feed ingredients can influence metabolic and proteomic processes in animals is crucial for evaluating their potential benefits and limitations.

Metabolomics and proteomics studies have become invaluable in animal science for understanding biological and physiological processes across species [22, 23]. Furthermore, Nuclear Magnetic Resonance (NMR) metabolomics techniques provide a comprehensive overview of animal physiology and production [24]. The integration of metabolomic and proteomic data helps elucidate connections between protein synthesis and metabolic pathways, offering explanations for complex biological phenomena that cannot be understood through single data sets alone [25].

Current knowledge about the effects of microalgae on the general health status and hepatic proteomics and metabolism of piglets is limited. Previous studies have considered proteomic approaches in porcine liver tissue. For instance, Cui et al. [26] investigated the hepatic proteomic response to heat stress in finishing pigs, while Lepczyński et al. [27] studied the effect of a chicory inulin extract on the hepatic proteome in growing pigs. Liu et al. [28] explored the impact of maternal folic acid supplementation on the hepatic proteome of new-born

piglets and, in the same line, Liu et al. [29] investigated the effect of intrauterine growth restriction on foetal pig hepatic proteome. Bondzio et al. [30] examined the influence of dietary zinc supplementation on the hepatic proteome of weaned piglets. In metabolomics, Lui et al. [31] studied the effect of meal frequency on plasma metabolite levels and hepatic proteome in pigs, while Yu et al. [32] integrated hepatic metabolomic and transcriptomic data to investigate sodium propionate infusion in a fistula pig model. Wang et al. [33] conducted hepatic metabolomic analyses in weaned piglets administered *Lactobacillus frumenti* or phosphate-buffered saline, and Parenti et al. [34] studied liver metabolomic changes due to severe acute malnutrition in piglets as a model for human conditions.

Our previous work explored the incorporation of *Chlorella vulgaris* at 5% and exogenous CAZyme supplementation in the diets of finishing pigs, revealing dietary influences on lipid metabolism and oxidative stress in the liver [35]. Drawing on these insights, we proposed that the combination of microalgae and CAZymes could enhance the health and metabolic condition of piglets during the weaning period. This study evaluated the dietary effects of 10% Spirulina, alone or in combination with lysozyme, on the hepatic metabolome and proteome. A companion study demonstrated its effects on blood health markers, immune function (leukocytes and immunoglobulins), and oxidative status (serum antioxidant markers and liver antioxidant diterpenes and carotenoids) [9].

Results

Growth performance

The growth performance data are presented here for contextual purposes. The inclusion of Spirulina in the diet negatively affected the growth performance of the piglets ($P < 0.05$). Additionally, the supplementation with lysozyme did not have a significant impact on growth, as there were no notable differences between the Spirulina (SP) and Spirulina plus lysozyme (SP + L) groups ($P > 0.05$). As illustrated in Fig. 1, the final weight of the piglets in the SP and SP + L groups decreased by 9.5%, and the feed conversion ratio (FCR) increased by 10.6% compared to the control group. No significant differences ($P > 0.05$) were observed in average daily feed intake (ADFI) among the experimental groups, indicating that the lower growth performance could be attributed to reduced nutrient digestibility in the SP-based diets. For comprehensive details of these results, please refer to our companion paper [11].

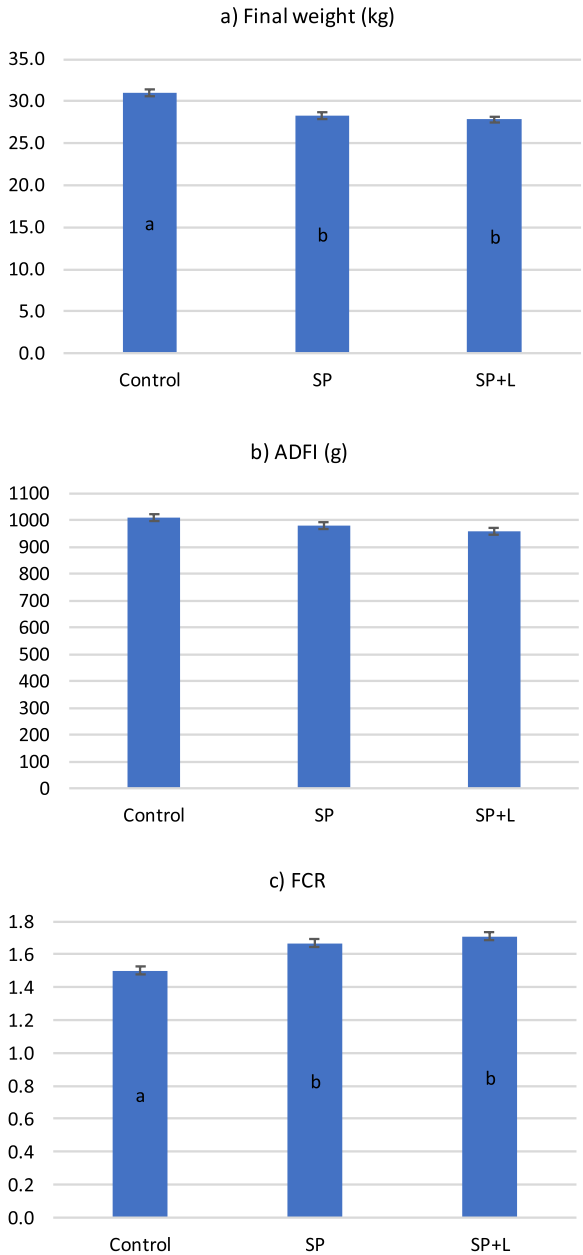


Fig. 1 Influence of experimental diets on: Final weight (**a**), ADFI (**b**) and FCR (**c**) of piglets. ADFI—Average daily feed intake; FCR—feed conversion ratio. Control—control diet; SP—10% Spirulina diet; SP + L—10% Spirulina diet supplemented with lysozyme..[a,b] Values within a row with different superscripts differ significantly at $P < 0.05$

Metabolomics results

Figure 2 displays the assignment of metabolites in a representative ^{1}H NMR spectrum of liver tissue. Sixty metabolites were identified and quantified in the liver tissue of piglets, listed in Table 1 by alphabetic order. Figures 3 and 4 schematically represent these identified metabolites, divided into high (values from 5×10^{-1} µmol/g tissue) and

low (values up to 5×10^{-1} µmol/g tissue) concentrations, respectively. Glucose and lactate were the most abundant metabolites in the liver tissue of piglets.

The analysis of the spectra revealed no widespread changes among the experimental groups (refer to Supplementary material—Figure S1). The spectra were very similar across all groups, except for a small number of compounds that appeared to be Spirulina-specific and could not be identified.

Figure 5 presents the Principal Components Analysis (PCA) and a supervised Partial Least Squares Discriminant Analysis (PLS-DA) of the hepatic metabolome. The PCA plot (Fig. 5A) suggests no global differences between the three experimental groups, indicating that the metabolic profiles were very similar. This observation aligns with the initial visual inspection of the individual NMR spectra. The PLS-DA analysis (Fig. 5B) was conducted to amplify the differences between the experimental groups, allowing for the detection of subtle changes. However, the statistical parameter Q2, which must exceed 0.4 for significant differences, was 0.28 for our results (see Supplementary material—Table S1). This value suggests minimal if any, global differences. There was a slight indication that the control group differed marginally from the other experimental groups. To further investigate these differences, we performed sparse PLS-DA (sPLS-DA) analysis (Fig. 6), which retains only the data showing significant variation. Despite this, the sPLS-DA results did not demonstrate a representative global difference between the groups.

Proteomics results

A total of 2,560 proteins were identified in the hepatic tissue of piglets, of which 2,012 had high protein FDR confidence as determined by SEQUEST HT. The PCA plot shown in Fig. 7 indicates no distinct clustering among the experimental groups, suggesting that the diets did not have a major impact on the hepatic proteome.

In the differential abundance analysis, 132 proteins were found to be significantly different between the Spirulina (SP) and control groups, 11 proteins between the Spirulina plus lysozyme (SP + L) and control groups, and 52 proteins between the SP + L and SP groups. The accession numbers in SwissProt database, descriptions, and gene symbols of these differentially abundant proteins are detailed in Tables 2, 3 and 4, respectively.

Figure 8 presents volcano plots for each comparison: SP *vs.* Control (A), SP + L *vs.* Control (B) and SP + L *vs.* SP (C). The Venn diagram in Fig. 9 shows that only 4 proteins were common between the SP + L *vs.* Control and SP *vs.* Control comparisons, and 9 proteins were common between the SP *vs.* Control and SP + L *vs.* SP comparisons.

Heatmaps illustrating the differential proteins for all experimental comparisons are presented in Fig. 10, showing no clear clustering. Additional heatmaps for each comparison (Fig. 11) also demonstrate no distinct clustering among the experimental groups.

The gene ontology classification of differentially abundant proteins is shown in Fig. 12. Detailed information about these proteins, categorized by molecular function, biological process and cellular component for different comparisons, can be found in the Supplementary material: SP *vs.* Control (Figure S2), SP + L *vs.* Control (Figure S3) and SP + L *vs.* SP (Figure S4). In short, the comparison SP + L *vs.* Control had more pathways involved in protein comparison (27), followed by the comparison of SP *vs.* Control (23), and finally SP + L *vs.* SP (10). Specifically, to these comparisons the highest number of genes were attributed to generation of precursor metabolites and energy, monocarboxylic acid metabolic and muscle system processes, and small molecule catabolic process, respectively.

The protein interaction networks for the differentially expressed proteins in the liver of piglets are displayed for the comparisons of SP *vs.* Control (Fig. 13), SP + L *vs.* Control (Fig. 14) and SP + L *vs.* SP (Fig. 15).

Discussion

The use of microalgae in animal feeding is an emerging area of research. Metabolomics and proteomics techniques can significantly enhance research in this field.

In addition to the current study, our previous research on the *longissimus lumborum* muscle provided valuable

(See figure on next page.)

Fig. 2 Representative 800 MHz ^{1}H 1D NOESY spectrum of liver aqueous fraction in piglets. Key: (1) 2-Hydroxyvalerate; (2) 4-Hydroxyphenyllactate; (3) 4-Pyridoxate; (4) acetate; (5) adenine; (6) adenosine; (7) ADP; (8) alanine; (9) allantoin; (10) AMP; (11) anserine; (12) arginine; (13) ascorbate; (14) asparagine; (15) aspartate; (16) ATP; (17) betaine; (18) choline; (19) creatine; (20) creatinine; (21) cytidine; (22) ethanolamine; (23) formate; (24) fumarate; (25) glucose; (26) glucose-6-phosphate; (27) glutamate; (28) glutamine; (29) glutathione; (30) glycerol; (31) glycine; (32) GTP; (33) guanosine; (34) histidine; (35) IMP; (36) inosine; (37) isoleucine; (38) lactate; (39) leucine; (40) lysine; (41) maltose; (42) mannose; (43) methanol; (44) methionine; (46) NAD+; (47) NADP+; (48) nicotinurate; (49) o-phosphocholine; (50) *o*-phosphoethanolamine; (51) phenylalanine; (52) proline; (53) riboflavin; (54) sarcosine; (55) serine; (56) sn-glycerol-3-phosphocholine; (57) succinate; (58) taurine; (59) threonine; (60) trigonelline; (61) tryptophan; (62) tyrosine; (63) UDP-galactose; (64) UDP-glucose; (65) UDP-glucuronate; (66) UDP-N-Acetylglucosamine; (67) UMP; (68) uracil; (69) uridine; (70) valine

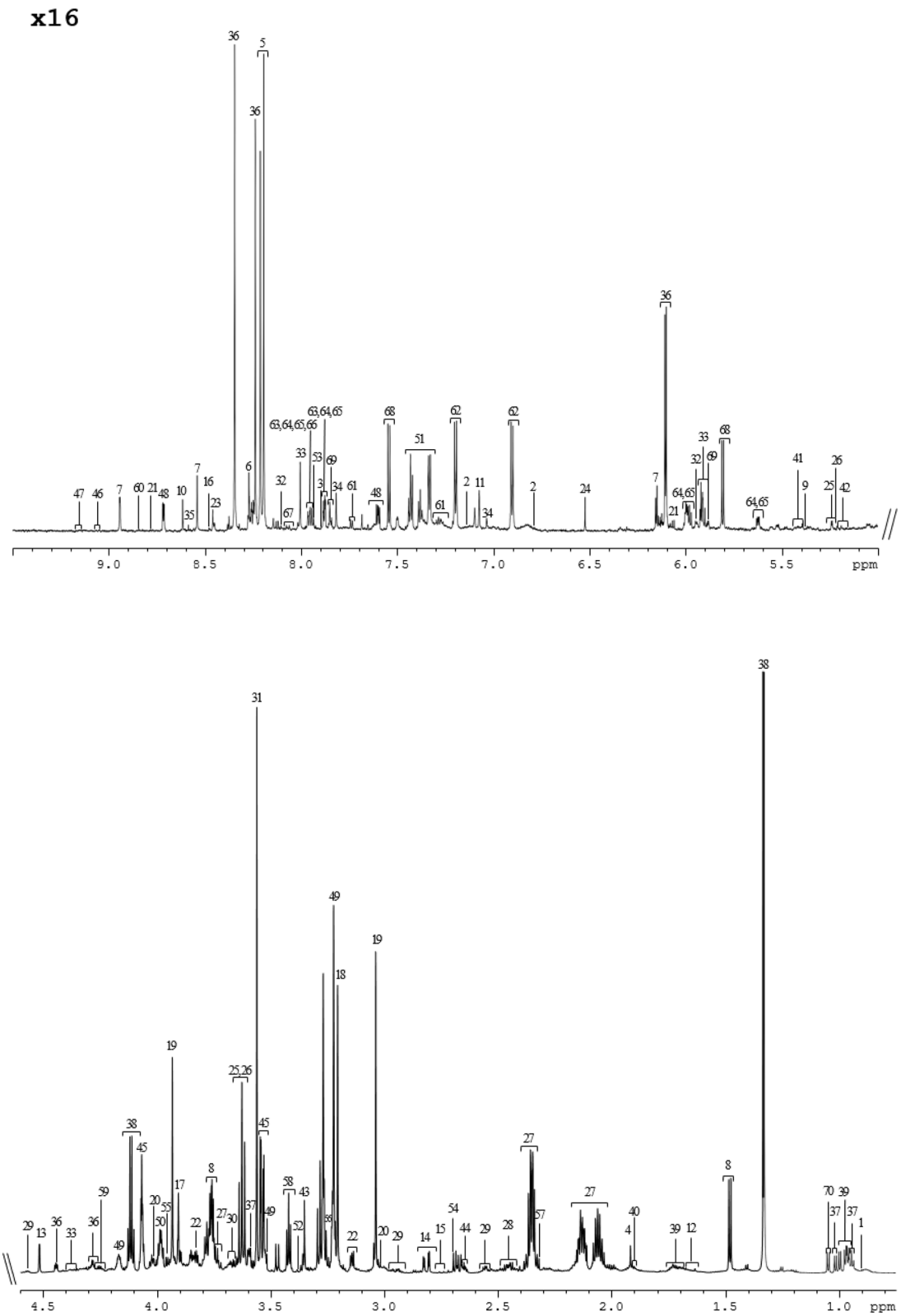


Fig. 2 (See legend on previous page.)

Table 1 Identified metabolites in aqueous fraction of liver tissue of piglets, organized by alphabetic order

Metabolite	(µmol/g tissue) [a]			Confidence level of signature [b]
4-Hydroxyphenyllactate	5.94×10^{-2}	±	1.0×10^{-2}	1
4-Pyridoxate	6.20×10^{-2}	±	9.40×10^{-3}	2
Acetate	1.26×10^{-1}	±	4.94×10^{-2}	4
Adenine	1.73×10^{-1}	±	4.58×10^{-2}	4
Adenosine Diphosphate	1.80×10^{-2}	±	8.56×10^{-3}	4
Alanine	1.24×10^{0}	±	4.14×10^{-1}	1
Allantoin	1.15×10^{-1}	±	2.31×10^{-2}	2
Adenosine Monophosphate	1.82×10^{-1}	±	5.93×10^{-2}	1
Anserine	9.79×10^{-2}	±	2.47×10^{-2}	2
Arginine	5.71×10^{-2}	±	1.36×10^{-2}	2
Ascorbate	5.96×10^{-1}	±	1.14×10^{-1}	4
Asparagine	1.19×10^{-1}	±	1.47×10^{-2}	2
Aspartate	5.90×10^{-1}	±	7.48×10^{-2}	2
Adenosine Triphosphate	2.28×10^{-2}	±	4.00×10^{-3}	1
Betaine	1.47×10^{0}	±	5.51×10^{-1}	2
Choline	1.68×10^{-1}	±	4.94×10^{-2}	2
Creatine	5.00×10^{-1}	±	2.91×10^{-1}	3
Creatinine	1.17×10^{-1}	±	5.02×10^{-2}	3
Cysteine	1.08×10^{-1}	±	2.64×10^{-2}	3
Glucose	2.13×10^{1}	±	4.31×10^{0}	2
Glucose-6-phosphate	4.46×10^{-1}	±	8.25×10^{-2}	3
Glutamate	1.15×10^{0}	±	2.93×10^{-1}	3
Glutamine	4.31×10^{-1}	±	6.27×10^{-2}	3
Glutathione	2.73×10^{-1}	±	1.09×10^{-1}	3
Glycerol	7.97×10^{-1}	±	1.33×10^{-1}	2
Glycine	1.09×10^{0}	±	1.70×10^{-1}	1
Histidine	4.80×10^{-2}	±	9.33×10^{-3}	1
Inosine Monophosphate	4.16×10^{-2}	±	1.30×10^{-2}	4
Inosine	6.20×10^{-1}	±	1.03×10^{-1}	1
Isoleucine	8.67×10^{-2}	±	1.61×10^{-2}	1
Lactate	6.68×10^{0}	±	1.35×10^{0}	1
Leucine	2.01×10^{-1}	±	3.72×10^{-2}	2
Lysine	4.77×10^{-2}	±	1.44×10^{-2}	4
Maltose	3.43×10^{-1}	±	6.81×10^{-2}	4
Mannose	3.00×10^{-1}	±	6.84×10^{-2}	1
Methanol	2.48×10^{-1}	±	1.47×10^{-1}	3
Methionine	4.95×10^{-2}	±	6.45×10^{-3}	2
NAD +	7.69×10^{-2}	±	2.22×10^{-2}	4
NADP +	3.20×10^{-2}	±	7.24×10^{-3}	4
Nicotinurate	2.32×10^{-1}	±	3.46×10^{-2}	1
O-Phosphocholine	2.07×10^{0}	±	6.68×10^{-1}	1
Phenylalanine	6.04×10^{-2}	±	9.25×10^{-3}	1
Proline	3.18×10^{-1}	±	4.06×10^{-2}	2
Riboflavin	2.09×10^{-2}	±	3.56×10^{-3}	4
Sarcosine	6.42×10^{-2}	±	1.59×10^{-2}	2
Serine	5.39×10^{-1}	±	8.43×10^{-2}	4

Table 1 (continued)

Metabolite	(µmol/g tissue) [a]			Confidence level of signature [b]
sn-Glycero-3-phosphocholine	3.52×10^{0}	±	1.25×10^{0}	2
Succinate	1.56×10^{-1}	±	4.97×10^{-2}	4
Taurine	2.77×10^{-1}	±	6.59×10^{-2}	3
Trigonelline	3.06×10^{-2}	±	1.46×10^{-2}	2
Tryptophan	1.80×10^{-2}	±	4.29×10^{-3}	1
Tyrosine	8.13×10^{-2}	±	2.01×10^{-2}	4
UDP-galactose	1.90×10^{-2}	±	9.78×10^{-3}	4
UDP-glucose	3.47×10^{-2}	±	1.74×10^{-2}	4
UDP-glucuronate	3.22×10^{-2}	±	7.30×10^{-3}	4
UDP-N-Acetylglucosamine	7.64×10^{-2}	±	1.60×10^{-2}	4
UMP	1.00×10^{-1}	±	2.67×10^{-2}	1
Uracil	4.48×10^{-2}	±	1.38×10^{-2}	1
Uridine	1.39×10^{-1}	±	3.52×10^{-2}	3
Valine	1.16×10^{-1}	±	2.11×10^{-2}	1

[a] Average concentration and standard deviation (µmol/g tissue)

[b] Confidence level of signature (1 to 4): 1 corresponds to signatures without measurement uncertainties; the subsequent levels correspond to a confidence level decreasing

insights into the effects of Spirulina dietary inclusion and lysozyme supplementation. While that study focused on muscle tissue, the findings support and complement the results presented here, particularly about nutrient metabolism and protein synthesis [13]. In that previous publication, we highlighted that glycogen metabolism and the utilization of nutrient reserves increased in the muscle of Spirulina-fed piglets. Additionally, the inclusion of lysozyme promoted muscle structural protein synthesis. Further related to this study, we published proteomic and metabolic results related to intestinal functions, associating these findings with gut histological results [15]. The inclusion of Spirulina and supplementation with lysozyme in piglet diets were correlated with changes in intestinal proteomics, notably increased protein synthesis and the abundance of contractile apparatus proteins. Such previous findings underscore the usefulness of proteomics in elucidating the effects of microalgae inclusion and lysozyme supplementation.

Given the complexity of the differences observed, we have divided our discussion into two parts: one focusing on the liver metabolome and the other on the liver proteome. Within the discussion of the proteomic results, due to their complexity and the differences identified, we further subdivided the analysis to provide a clearer understanding of the findings.

Hepatic metabolome

In general, the metabolomics data did not reveal a clear separation between the three experimental groups. We hypothesized that hepatic metabolism might show significant changes that could explain the differences observed in the growth performance of the animals, as the Spirulina groups exhibited impaired growth. The liver is a key organ for lipid metabolism, and hepatic lipid levels can be influenced by several pathways. However, it is challenging to pinpoint the specific mechanisms by which nutrients regulate hepatic lipid metabolism. Metabolomics analyses, which examine various metabolites from biological samples, provide an overview of metabolic changes related to dietary intake [36].

Despite the lack of significant differences between the experimental groups, we proceeded with the assignment and identification of metabolites. Identifying and quantifying sixty metabolites in piglet liver tissue associated with the new diets could provide a comprehensive view of metabolic composition. Glucose and lactate were the most abundant metabolites identified in the liver of piglets, consistent with previous literature describing these metabolites in healthy porcine liver tissue [37, 38].

In a previous study by our research team, the dietary incorporation of 5% *Chlorella vulgaris* in piglet diets resulted in the hepatic NMR-metabolomics analysis identifying 28 metabolites, including glucose, creatinine and lactate [14]. The results of that study indicated similar final metabolome profiles (general composition

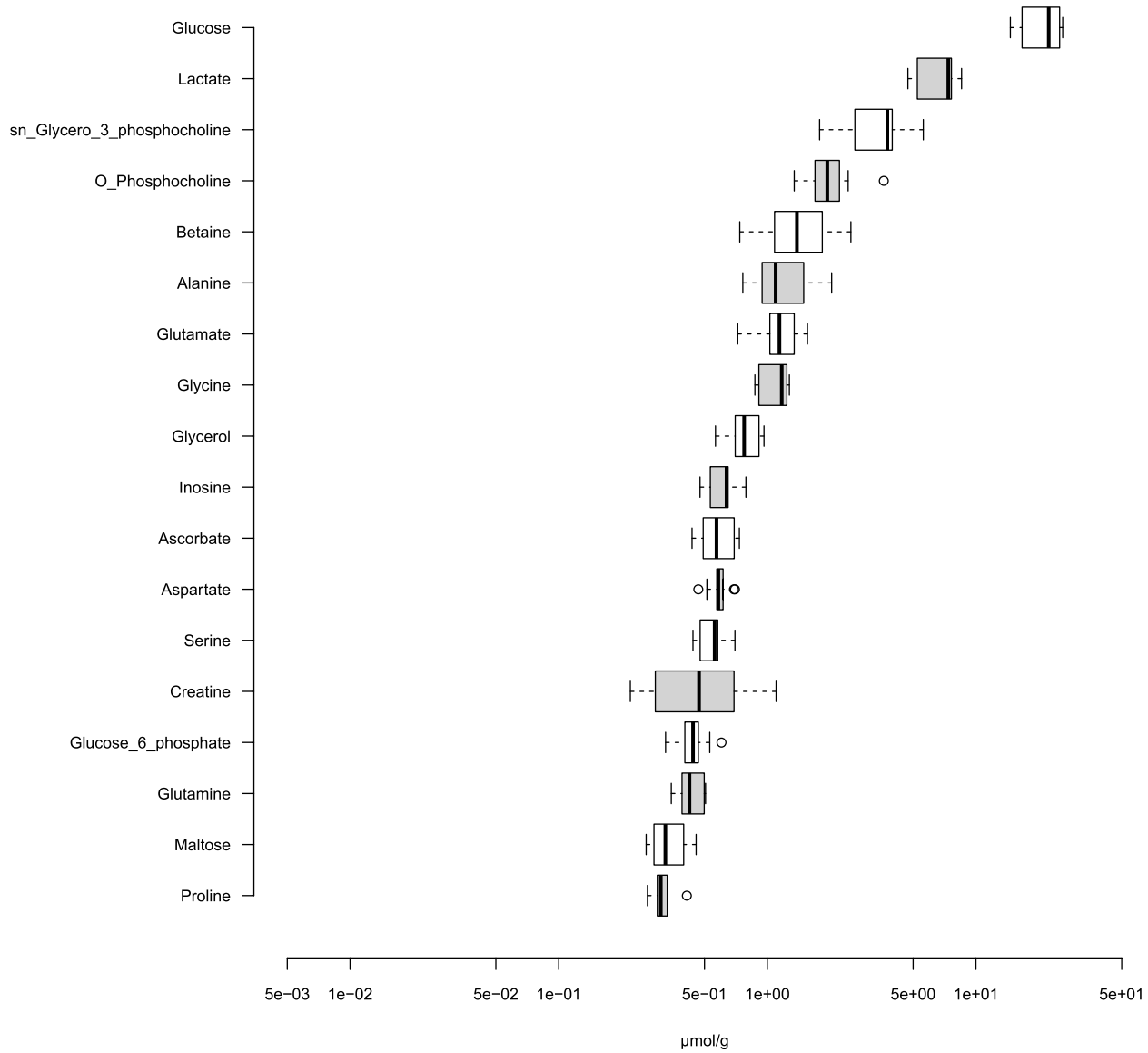


Fig. 3 Box plot of identified higher concentration metabolites in aqueous fraction of liver tissue of piglets

and concentration) among all experimental groups, suggesting that the incorporation of *Chlorella vulgaris* in diets, supplemented or not with feed enzymes, has residual effects on overall hepatic intermediary metabolism [14]. These findings are consistent with the current study, where we observed only minor differences in the liver metabolome. In the *Chlorella vulgaris* trial, no differences in growth performance were observed, whereas in this study, the Spirulina-fed piglets showed limited growth compared to the control group. Li et al. [39] confirmed that amino acid metabolism is highly associated with dietary protein restriction through intestinal and liver metabolomics. In our study, the diets were balanced for protein content, so the absence of significant metabolic differences aligns with this context.

Hepatic proteome—SP *vs.* Control: The effect of dietary Spirulina incorporation

All proteins identified as differentially expressed between the control and SP groups were found in greater abundance in the SP group. Most of these proteins were involved in several metabolic process's characteristic of liver function, such as nucleoside phosphate biosynthesis, steroid metabolism, cofactor

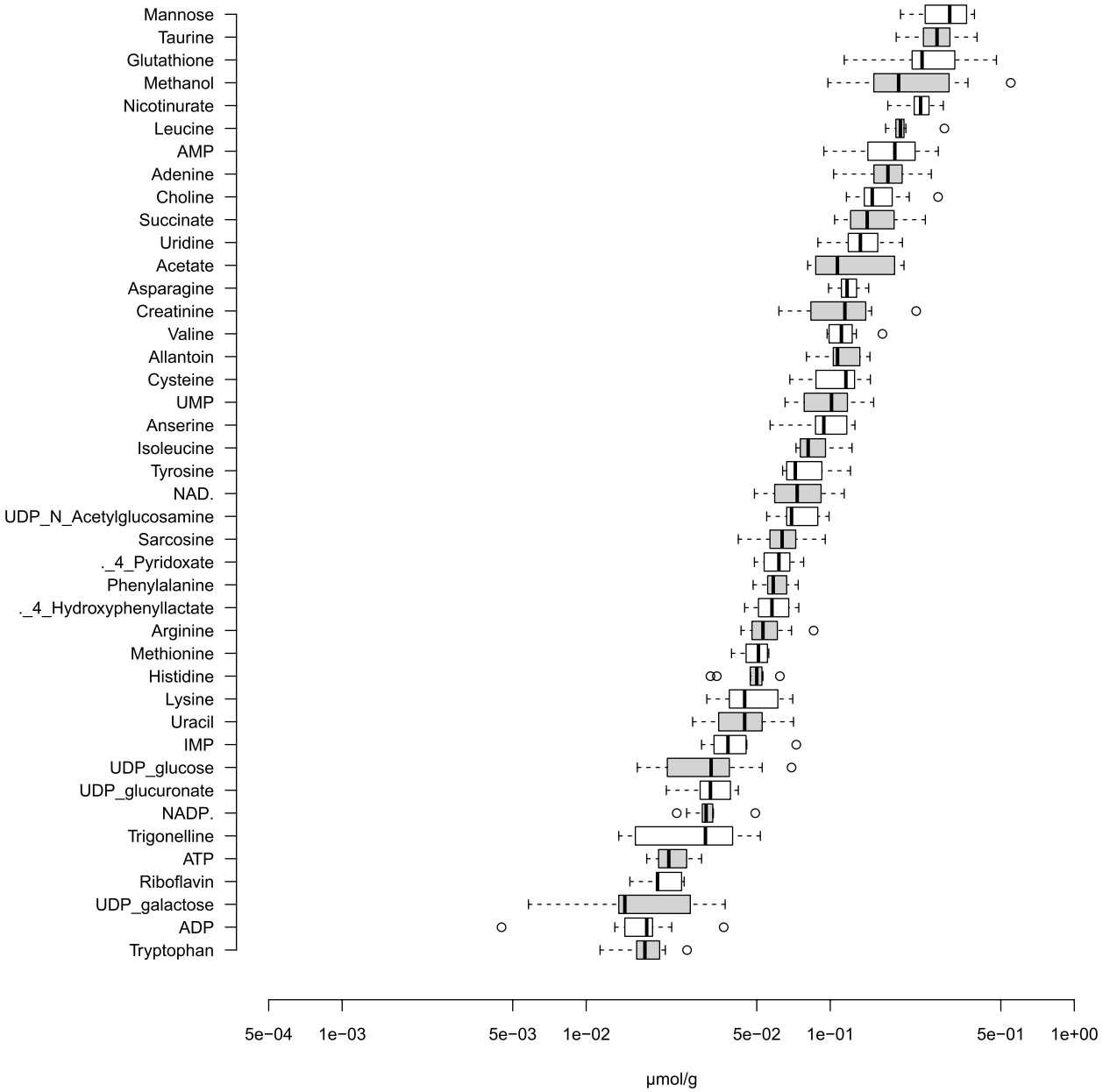


Fig. 4 Box plot of lower identified metabolites in aqueous fraction of liver tissue of piglets

biosynthesis, lipid modification and response to metal ions.

Bondzio et al. [30] reported that high zinc levels upregulate metallothionein in the liver, which is a key protein in regulating cellular zinc homeostasis. In our study, the Spirulina diet had higher zinc levels than the control diet (141 vs. 129 mg/kg preliminary results, data not published), which likely contributed to the observed upregulation of metallothionein. Zinc plays a critical role in numerous biological processes, including enzyme function, immune response, and cellular repair mechanisms. The increased zinc levels in the Spirulina-fed group could be attributed to the high mineral content of Spirulina, which may lead to a physiological adaptation aimed at maintaining zinc balance. This upregulation of metallothionein not only aids in zinc homeostasis but also provides a protective mechanism against oxidative stress, highlighting the dual role of this protein in both detoxification and cellular defence. Metallothionein is a low molecular weight protein rich in cysteine that binds

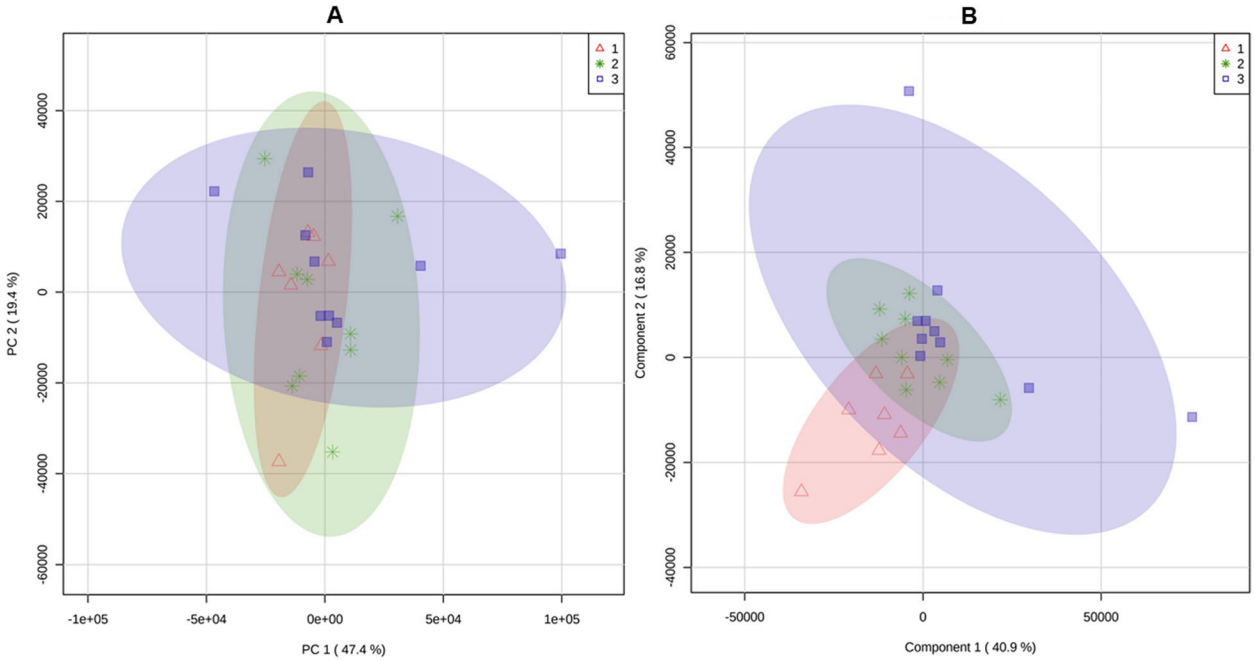


Fig. 5 PCA (**A**) and PLS-DA (**B**) scatterplot of liver metabolite composition for the three experimental groups. PCA—Principal Components Analysis; PLS-DA—Partial Least Squares Discriminant Analysis. 1—Control; 2—10% Spirulina diet (SP); 3—10% Spirulina diet supplemented with lysozyme (SP + L)

essential metals like zinc and regulates cellular zinc levels. Its induction by dietary zinc levels and its role in regulating zinc signalling in immune responses highlight its importance in maintaining healthy cellular processes [40], thus explaining our results. In these young animals, zinc balance is a delicate mechanism that can be associated with deficiency problems leading to immunosuppression or, on the other hand, its excess can cause toxicity. The role of metallothionein in zinc homeostasis at this young stage of the animal may be crucial due to its beneficial effect on the immune system.

Heat shock proteins (HSP) play an important role in protecting cells from damage caused by stress, including responses to heat or other stress factors, and are involved in immune defence [41]. Cui et al. [26] found an increased abundance of proteins and enzymes associated with the heat shock response in the liver of finishing pigs exposed to chronic heat stress. Our study also found an increase in HSP levels (heat shock protein) in the SP group compared to the control group. Although these animals were not exposed to differential heat stress, it is possible that the physiological stress induced by the dietary inclusion of Spirulina triggered the upregulation of these proteins, similar to responses observed under heat stress. Previous studies have demonstrated that certain dietary supplements can induce physiological stress in animals, leading to increased expression of stress-related proteins such as heat shock proteins (HSPs). For example, Yun et al. [42] showed that dietary arginine supplementation mitigated reductions in average daily gain and feed efficiency in weaned piglets exposed to heat stress, suggesting a strong link between dietary interventions and stress responses. Similarly, stress induced by dietary Spirulina inclusion in our study may have activated the upregulation of HSPs as part of a protective cellular response. Carboxylesterases (CE, EC 3.1.1.1) are crucial hydrolases with remarkable catalytic activity, hydrolysing carboxylic acid esters and amides. They are central to drug metabolism and pesticide detoxification [43]. Pigs have multiple carboxylesterase genes, with higher liver expression levels than other mammals, earning them the name pig liver esterases (PLE). Carboxylesterases have shown impressive enantio- and stereoselectivity, making them valuable in biosynthesis [44, 45]. The antioxidant properties of peptides derived from carboxylic acid ester hydrolase and pig liver keratin were highlighted by López-Pedrouso et al. [46]. These peptides act as antioxidants by donating hydrogen or electrons to neutralise free radicals or by chelating metals to prevent oxidation reactions [47]. The potential use of porcine liver hydrolysates has been investigated, but further studies are needed to prove their bioaccessibility. In our study, the hepatic tissue of the SP groups contained higher levels of these proteins than the control group, potentially indicating a physiological response to

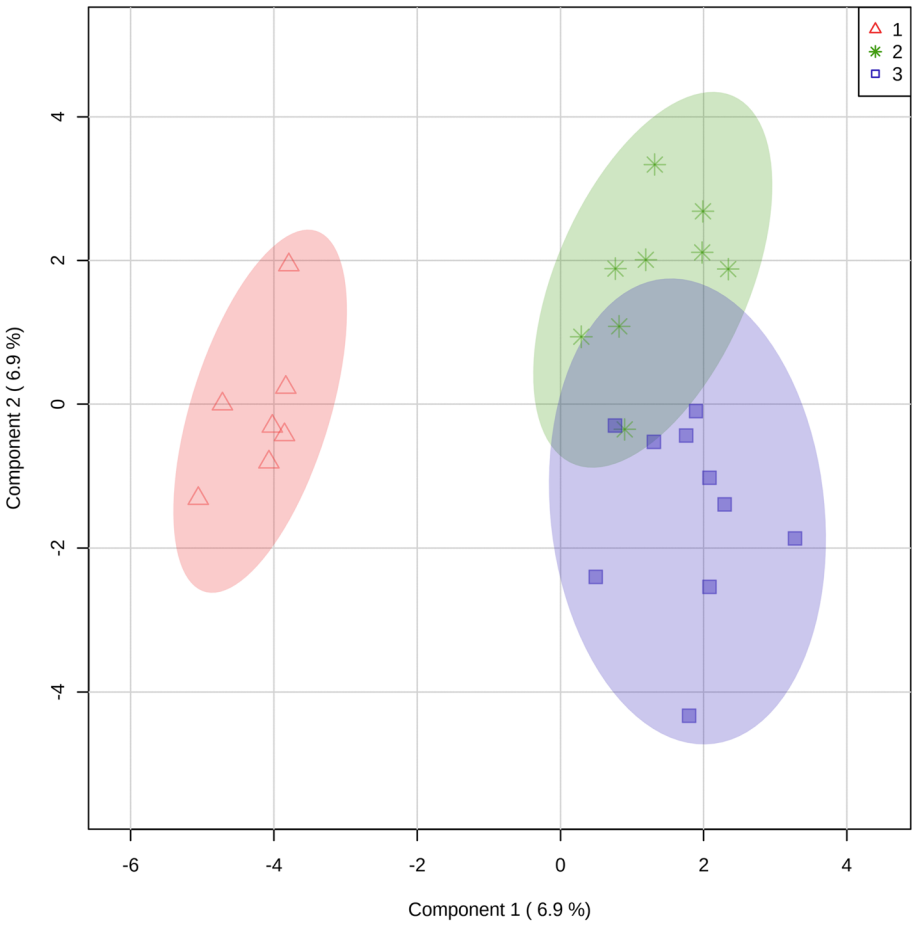


Fig. 6 sPLS-DA scatterplot of liver metabolite composition for the three experimental groups sPLS-DA—Sparce Partial Least Squares Discriminant Analysis. 1—Control; 2—10% Spirulina diet (SP); 3—10% Spirulina diet supplemented with lysozyme (SP + L)

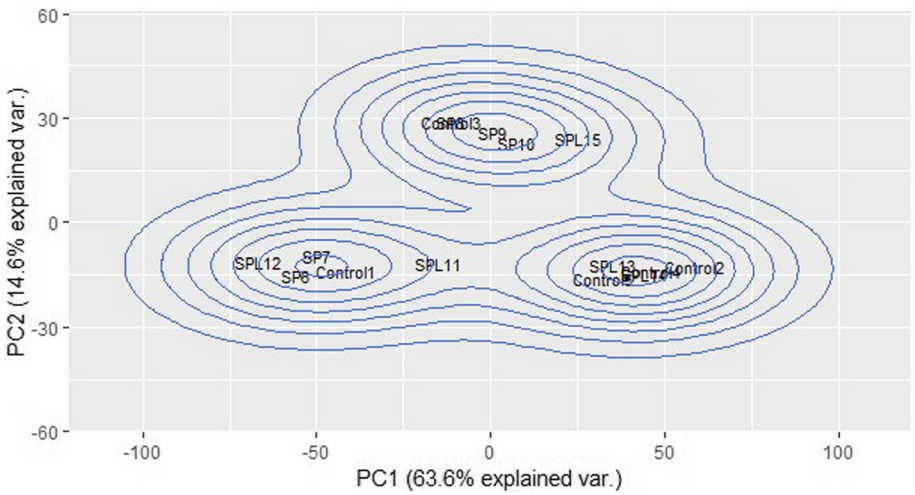


Fig. 7 PCA of quantified proteins from the liver of piglets fed with the three experimental diets. PCA -Principal Component Analysis. Control - control diet; SP - 10% Spirulina diet; SP+L - 10% Spirulina diet supplemented with lysozyme

Table 2 Liver proteins showing differential expression in piglets fed SP and Control diets

Accession number in SwissProt database	Protein description [*Sus scrofa*]	Gene Symbol	PE	SV	*p*-value	Log2 FC (SP *vs*. Control)
A5GFY6	Novel protein similar to vertebrate phosphoglycerate dehydrogenase (PHGDH) (Fragment)	CH242-38B5.3–001	4	1	0.0159	-0.8389
A0A286ZLH1	Uncharacterized protein	PSMD2	1	1	0.0159	-0.8013
F1SGG1	Keratin 18	KRT18	1	3	0.0079	-0.7662
F1S0K2	Keratin 15	KRT15	1	2	0.0317	-0.6886
I3LB63	Uncharacterized protein	LOC100518109	1	2	0.0465	-0.6869
A0A1B2TT84	Cytochrome P450 subfamily IIIA polypeptide 22 isoform 2	CYP3A22	2	1	0.0079	-0.6530
I3LA57	Cytochrome P450 3A29	CYP3A29	1	2	0.0079	-0.6530
A0A286ZYF3	Metallothionein	MT1D	3	1	0.0079	-0.6441
F1RQQ7	Alpha-1,4 glucan phosphorylase	PYGB	1	3	0.0195	-0.6395
F1RQQ8	Alpha-1,4 glucan phosphorylase	PYGM	1	2	0.0159	-0.6360
A0A287A4X5	Uncharacterized protein	LOC110261008	3	1	0.0159	-0.6343
A0A287AZ82	Uncharacterized protein	KRT8	1	1	0.0317	-0.6175
F1SKJ1	Myosin heavy chain 9	MYH9	1	3	0.0079	-0.5963
A0A287AID2	Tropomyosin alpha-3 chain	TPM3	1	1	0.0079	-0.5908
Q5IZV0	Heat shock protein 27 kDa (Fragment)	N.D	2	1	0.0159	-0.5906
F1RRF2	STT3B, catalytic subunit of the oligosaccharyltransferase complex	STT3B	1	1	0.0159	-0.5810
F1SDB7	Flavin-containing monooxygenase	FMO5	1	2	0.0079	-0.5685
F1SKI0	Myosin-11	MYH11	1	3	0.0119	-0.5604
K9J6M1	Ras GTPase-activating-like protein IQGAP2	IQGAP2	2	1	0.0159	-0.5434
A0A1S6L948	Carboxylic ester hydrolase	PLE-C4	3	1	0.0159	-0.5422
I3LV99	Myosin heavy chain 14	MYH14	1	2	0.0119	-0.5418
A0A287BQU7	Small nuclear ribonucleoprotein Sm D2	SNRPD2	1	1	0.0357	-0.5396
A0A287AGA4	6-phosphofructokinase	PFKL	1	1	0.0159	-0.5361
F1SCD0	Uncharacterized protein	LOC396685	1	1	0.0208	-0.5351
A0A2C9F3D9	Vimentin	VIM	1	1	0.0357	-0.5293
A0A286ZX11	Hypoxia up-regulated 1	HYOU1	1	1	0.0357	-0.5261
A0A287AQV4	Cystathionine beta-synthase	CBS	3	1	0.0079	-0.5216
I3LLT2	Uncharacterized protein	CAD	1	2	0.0159	-0.5048
A0A286ZXQ0	Atlastin GTPase 3	ATL3	1	1	0.0212	-0.5018
D3K5K2	Peptidylprolyl isomerase	FKBP4	2	1	0.0079	-0.5007
A0A287AWK8	High density lipoprotein binding protein	HDLBP	1	1	0.0317	-0.4950
A0A287AAJ9	Acyl-coenzyme A oxidase	ACOX2	1	1	0.0317	-0.4949
A0A287B9G0	Aldehyde dehydrogenase	ALDH3A2	1	1	0.0317	-0.4906
A0A287B7R3	Prostaglandin reductase 1	PTGR1	4	1	0.0358	-0.4897
A0A287ACJ9	Metallothionein	MT1A	3	1	0.0465	-0.4859
A0A287AQU3	Uncharacterized protein	XDH	1	1	0.0357	-0.4854
A0A287A2N6	MICOS complex subunit MIC60	IMMT	1	1	0.0079	-0.4716
B1A8Z3	Alpha-1,4 glucan phosphorylase	PYGL	2	1	0.0189	-0.4701
D2D0E2	Carnitine palmitoyltransferase II	CPT2	3	1	0.0317	-0.4670
A5D9N3	Allograft inflammatory factor 1	AIF1	1	1	0.0159	-0.4665
A0A287AII8	1,4-alpha-glucan branching enzyme 1	GBE1	1	1	0.0497	-0.4647
F1RF46	Asialoglycoprotein receptor 2	ASGR2	1	3	0.0079	-0.4636
F1S0K1	Keratin 13	KRT13	1	2	0.0317	-0.4632
D0G6Y1	Hydroxysteroid (17-beta) dehydrogenase 2	HSD17B2	2	1	0.0159	-0.4623
B7TJ02	Thymosin beta 4 X-linked	TMSB4X	2	1	0.0317	-0.4594
K9IVW4	Myotrophin	MTPN	1	1	0.0159	-0.4588
A0A0B8RTA8	Acyl-CoA dehydrogenase, very long chain	ACADVL	3	1	0.0212	-0.4541

Table 2 (continued)

Accession number in SwissProt database	Protein description [*Sus scrofa*]	Gene Symbol	PE	SV	*p*-value	Log2 FC (SP *vs*. Control)
I3LDY5	Lactate dehydrogenase D	LDHD	1	2	0.0317	-0.4457
F1RMV5	ETHE1, persulfide dioxygenase	ETHE1	1	2	0.0317	-0.4449
F1SCF0	Alpha-1-antitrypsin	SERPINA1	3	2	0.0159	-0.4410
F1SMW9	Proteasome 26S subunit, non-ATPase 1	PSMD1	1	3	0.0159	-0.4383
A0A068CA64	Mitochondrial apoptosis-inducing factor 1 transcript variant 1	AIFM1	2	1	0.0465	-0.4368
F1SB81	Plasminogen	PLG	1	2	0.0079	-0.4358
A0A287ASR0	Uncharacterized protein	SLC25A10	3	1	0.0212	-0.4357
A0A287A9H4	Uncharacterized protein	HNRNPF	1	1	0.0159	-0.4326
I3LKM9	Legumain	LGMN	1	2	0.0317	-0.4325
F1RH12	Uncharacterized protein	ASPDH	1	1	0.0317	-0.4306
Q9GLE9	Heat shock 90kD protein 1, beta (Fragment)	HSPCB	2	1	0.0119	-0.4251
I3LNT8	Solute carrier family 2, facilitated glucose transporter member 2	SLC2A2	1	2	0.0159	-0.4250
B2CZR7	CFL2b variant 1	CFL2b	2	1	0.0317	-0.4243
B9W5V0	Elongation factor 1-alpha	EEF1A2	3	1	0.0212	-0.4206
F1SCT4	Cytochrome P450 2E1	CYP2E1	1	1	0.0361	-0.4191
F1ST81	Drebrin like	DBNL	1	2	0.0361	-0.4171
F1STM4	Elongation factor 1-alpha	LOC100514912	3	1	0.0196	-0.4143
A1BPP7	Beta actin (Fragment)	ACTB	3	1	0.0273	-0.4129
A0A287BAJ3	PDZ domain containing 1	PDZK1	1	1	0.0159	-0.4096
A0A287AG15	Penta-EF-hand domain containing 1	PEF1	1	1	0.0117	-0.4077
A0A286ZZ49	Phytanoyl-CoA 2-hydroxylase	PHYH	1	1	0.0157	-0.4068
A0A287A4L3	Uncharacterized protein	GFPT1	1	1	0.0159	-0.4065
A0A286ZVL0	Heterogeneous nuclear ribonucleoprotein U	HNRNPU	1	1	0.0317	-0.4064
F1S6D8	UMP-CMP kinase	CMPK1	3	3	0.0159	-0.4043
I3LDQ1	Talin 2	TLN2	1	2	0.0212	-0.4025
A5GFS4	Phosphoenolpyruvate carboxykinase 1	PCK1	1	1	0.0317	-0.3997
A0A286ZM06	Choline dehydrogenase	CHDH	1	1	0.0160	-0.3987
F1SRJ8	Phenylalanine hydroxylase	PAH	1	3	0.0278	-0.3982
F1SNZ7	Succinate-CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial	SUCLG1	3	2	0.0079	-0.3974
F1RMN7	Hemopexin	HPX	3	3	0.0159	-0.3960
A0A287BDV9	Glycerol-3-phosphate dehydrogenase [NAD^+]	GPD1	1	1	0.0317	-0.3951
F1RRM1	2′-deoxynucleoside 5′-phosphate N-hydrolase 1	DNPH1	1	1	0.0317	-0.3933
B9TSR5	Valyl-tRNA synthetase	VARS	2	1	0.0262	-0.3911
A5A8V9	Valyl-tRNA synthetase 2	VARS2	3	1	0.0262	-0.3911
F1SKQ0	DEAD-box helicase 17	DDX17	1	3	0.0491	-0.3867
Q19QT8	Quinone oxidoreductase	CRYZ	2	1	0.0159	-0.3843
K7GNY4	Uncharacterized protein	RPL8	1	2	0.0195	-0.3794
F6QA08	Protein disulfide-isomerase	PDIA3	1	1	0.0357	-0.3793
Q2YHQ3	Filamin-A (Fragment)	FLNA	1	1	0.0079	-0.3702
B5KJG2	Phosphoglycerate mutase	PGAM2	1	1	0.0317	-0.3675
Q15B97	Adenylate kinase 3-like protein 1	AK3L1	2	1	0.0268	-0.3667
F1SBI1	Sorting nexin	SNX5	1	3	0.0361	-0.3663
B3CL06	Transferrin	TF	3	1	0.0159	-0.3638
E1U318	Glutamine:fructose-6-phosphate amidotransferase 1 variant 2	GFAT1	2	1	0.0286	-0.3630
A0A287BB01	Reticulon	RTN3	1	1	0.0317	-0.3626
F1SPF9	Coatomer subunit gamma	COPG1	1	3	0.0159	-0.3604
I3L804	Tyrosine–tRNA ligase	YARS	1	2	0.0212	-0.3574
A0A286ZQ44	Dicarbonyl and L-xylulose reductase	DCXR	4	1	0.0361	-0.3556

Table 2 (continued)

Accession number in SwissProt database	Protein description [*Sus scrofa*]	Gene Symbol	PE	SV	*p*-value	Log2 FC (SP *vs*. Control)
A0A287AZ17	Hydroxyacylglutathione hydrolase	HAGH	1	1	0.0317	-0.3536
Q6RVD5	Fatty acid coenzyme A ligase long-chain 2 (Fragment)	FACL2	4	1	0.0294	-0.3518
A0A287ANX1	Tetratricopeptide repeat domain 38	TTC38	1	1	0.0159	-0.3468
K9J4R3	Alpha-mannosidase	MAN2B1	2	1	0.0159	-0.3441
F1RYS9	Uncharacterized protein	FAM47E	1	2	0.0361	-0.3434
P79309	Tropomyosin TM30-pl (Fragment)	TNNT1	2	1	0.0079	-0.3425
A0A287ACK6	Uncharacterized protein	N.D	1	1	0.0278	-0.3409
A0A287BJS1	Eukaryotic translation initiation factor 4B	EIF4B	4	1	0.0317	-0.3394
D0G7F7	Tropomyosin 4	TPM4	2	1	0.0278	-0.3363
I3LEI8	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	ADI1	1	1	0.0195	-0.3359
A0A287A1G4	PDZ and LIM domain 5	PDLIM5	1	1	0.0159	-0.3349
A0A287AFH8	LIM domain 7	LMO7	1	1	0.0195	-0.3330
A0A286ZP76	Uncharacterized protein	AHNAK	1	1	0.0278	-0.3330
A0A0B8RZ32	Stress-induced phosphoprotein 1	STIP1	4	1	0.0159	-0.3329
I3LP02	Acetyl-CoA acetyltransferase 1	ACAT1	1	2	0.0317	-0.3304
A5X5D1	Nucleoside diphosphate kinase (Fragment)	NME1	3	1	0.0317	-0.3225
Q6QA71	Methylenetetrahydrofolate dehydrogenase (Fragment)	MTHFD1	2	1	0.0361	-0.3149
F1RX37	Fibrinogen beta chain	FGB	1	2	0.0212	-0.3138
J9JIK8	Protein-L-isoaspartate O-methyltransferase	PCMT1	3	2	0.0159	-0.3109
F1SBS4	Complement C3	C3	1	3	0.0278	-0.3079
I3LIZ8	Uncharacterized protein	LOC100739087	4	1	0.0465	-0.3078
A0A287AX39	60S ribosomal protein L36a	RPL36A-HNRNPH2	3	1	0.0159	-0.3036
A0A287AT58	Uncharacterized protein	SEC14L2	1	1	0.0286	-0.3035
K7GL74	Uncharacterized protein	PRPS2	1	2	0.0286	-0.3031
A0A0B8RZH4	Isoleucyl-tRNA synthetase 2, mitochondrial	IARS2	3	1	0.0459	-0.3031
F1SA44	Uncharacterized protein	GPHN	1	3	0.0317	-0.3011
A0A287BJ16	Uncharacterized protein	VPS26B	1	1	0.0119	-0.2997
F1S340	Uncharacterized protein	SRI	1	1	0.0465	-0.2951
A0A287A480	Coatomer subunit delta	ARCN1	1	1	0.0159	-0.2949
A0A287BD60	Tripeptidyl peptidase 2	TPP2	1	1	0.0189	-0.2943
A0A287B088	Proteasome subunit beta	PSMB4	1	1	0.0119	-0.2941
A0A2C9F345	U1 small nuclear ribonucleoprotein A	SNRPA	4	1	0.0317	-0.2895
Q9TTN8	Pyruvate kinase (Fragment)	PKLR	4	1	0.0317	-0.2831
F1SML8	40S ribosomal protein S6	RPS6	3	2	0.0317	-0.2587
A0A286ZIS0	GDP-mannose 4,6-dehydratase	GMDS	1	1	0.0361	-0.2562
A0A287BQI9	Uncharacterized protein	LOC100627489	4	1	0.0317	-0.2454
F2Z594	High mobility group protein B1	HMGB1	1	2	0.0317	-0.2167

PE Protein existence, *SV* Sequence version, *FC* Fold change, *N.D* No data Dietary treatment, *SP* Spirulina diet

The table does not show the redundant proteins—to see all, please see Supplementary material—FileS1

the stress caused by Spirulina inclusion, in line with findings for HSP.

Aldehyde dehydrogenase (ALDH) is a key enzyme in the ethanol catabolic pathway, converting toxic acetaldehyde to acetate. Yan et al. [48] linked the upregulation of ALDH to arginine supplementation in hepatocellular carcinoma cells. In our study, the dietary amino acid profiles, including arginine, were balanced, so the upregulation observed in the SP group may not be directly related to amino acid levels but could indicate a broader metabolic adjustment to dietary changes.

Plasminogen is crucial in the fibrinolytic system as the precursor of plasmin, which breaks down blood clots. Junghans et al. [49] found plasminogen expression

Table 3 Liver proteins showing differential expression in piglets fed SP + L and Control diets

Accession number in SwissProt database	Protein description [*Sus scrofa*]	Gene Symbol	PE	SV	*p*-value	Log2 FC (SP + L *vs.* Control)
I3LL15	Uricase	UOX	3	2	0.0159	-0.4557
B1NI70	Adenylate kinase 4, mitochondrial	AK3L1	1	1	0.0159	-0.4227
A5GFS4	Phosphoenolpyruvate carboxykinase 1	PCK1	1	1	0.0317	-0.3952
Q76N65	Cytochrome P450	CYP3A22	2	1	0.0079	-0.3516
F1RUY9	Carnosine synthase 1	CARNS1	1	2	0.0317	-0.3024
A0A287A608	Uncharacterized protein	AHNAK	1	1	0.0357	-0.2780
F1RX36	Uncharacterized protein	FGA	1	3	0.0459	-0.2774
F1SCF0	Alpha-1-antitrypsin	SERPINA1	3	2	0.0286	-0.2610
A0A286ZM06	Choline dehydrogenase	CHDH	1	1	0.0317	-0.2432
F1SK65	1,4-alpha-glucan branching enzyme 1	GBE1	1	3	0.0491	-0.2348
K9J4R3	Alpha-mannosidase	MAN2B1	2	1	0.0159	-0.2105

PE Protein existence, *SV* Sequence version, *FC* Fold change, Dietary treatments, *SP + L* Spirulina diet supplemented with lysozyme

The table does not show the redundant proteins—to see all, please see Supplementary material—FileS2

modulated by diet. In our study, despite isoproteic diets, the availability of nutrients was negatively impacted by Spirulina inclusion [11], which could explain the observed increase in plasminogen levels.

Cytochrome P450 (CYP450) enzymes, particularly CYP2E1, are vital for drug metabolism. In pigs, this enzyme is very similar to the human version (a sequence homology of 62 to 87.5%) and can be used as a tool to help scientists develop new medicines and ensure that they are safe for us [50]. In the SP group, we observed an upregulation of CYP2E1, a key enzyme involved in the metabolism of various drugs and xenobiotics. This increase could be a response to the introduction of Spirulina, as the liver upregulates enzymes to process the bioactive compounds present in the diet. The heightened expression of CYP2E1 suggests that Spirulina inclusion may influence hepatic detoxification pathways, which could have implications for drug metabolism in pigs. Testing the interaction of Spirulina supplementation with pharmaceutical compounds might be of interest to better understand how dietary supplements can alter drug efficacy or safety. This warrants further investigation to explore the potential effects of such dietary changes on the pharmacokinetics of drugs commonly administered to pigs [50, 51].

Protein disulphide-isomerase (PDIA3) plays crucial roles in electron carrier and cysteine-type endopeptidase activities [52]. Zhao et al. [53] identified PDIA3 as a novel target for the treatment of liver fibrosis in mice. These authors established that a downregulation of this protein was associated with liver fibrosis, reinforcing the idea that further studies on this protein will expand our understanding of the mechanisms of liver fibrogenesis and help to develop new therapeutic approaches for liver fibrosis. In our study, we found an up-regulation of this protein with Spirulina supplementation, which could be further investigated to determine if it could have the same significance in pigs, possibly associating this microalgae supplementation with a lower prevalence of liver fibrosis, as found by the aforementioned authors.

Transferrin, an iron-binding transport protein, plays a critical role in iron homeostasis [54]. Wang et al. [55] linked decreased transferrin levels to intrauterine growth restriction. Our study found upregulated transferrin in the SP group, indicating a potential disturbance in iron metabolism due to mineral imbalances from Spirulina supplementation, which aligns with findings of normal iron levels in piglets without anaemia.

Complement C3, a key component of the immune response, was upregulated in the SP group, indicating an activation of the complement cascade and a stress response to dietary Spirulina inclusion. Wang et al. [56] also observed the involvement of the complement system in the liver's response to stress and injury. At the macro level, necropsy results revealed no signs of liver damage or abnormalities in any of the experimental groups, including those fed Spirulina. Additionally, there were no significant differences in liver weight between the groups ($P = 0.1331$), with liver weights averaging 22.8 g/kg live weight across all groups. These findings suggest that the observed upregulation of certain proteins, such as PDIA3, in the SP group is more likely a reflection of a physiological adaptation to Spirulina supplementation rather than an indication of tissue damage. Proteasome subunit beta was upregulated in the SP group, suggesting increased protein degradation processes, potentially

Table 4 Liver proteins showing differential expression in piglets fed SP + L and SP diets

Accession number in SwissProt database	Protein description [*Sus scrofa*]	Gene Symbol	PE	SV	*p*-value	Log2 FC (SP + L *vs.* SP)
A0A286ZXQ5	Uncharacterized protein	LOC100626015	1	1	0.0497	-0.0813
A0A0B8RZH4	Isoleucyl-tRNA synthetase 2, mitochondrial	IARS2	3	1	0.0459	-0.2199
F1SFI7	Alpha-2-HS-glycoprotein	AHSG	1	3	0.0317	-0.2534
A0A287BMV2	Serine/arginine-rich-splicing factor 1	SRSF1	1	1	0.0317	-0.2641
A0A287BQI5	Phytanoyl-CoA 2-hydroxylase	PHYH	1	1	0.0212	-0.2826
Q9TTN8	Pyruvate kinase (Fragment)	PKLR	4	1	0.0361	-0.2831
A0A287A675	Cystathionine beta-synthase	CBS	3	1	0.0079	-0.2832
A0A287AG15	Penta-EF-hand domain containing 1	PEF1	1	1	0.0195	-0.2851
A0A287A2N6	MICOS complex subunit MIC60	IMMT	1	1	0.0079	-0.2935
A0A287A6V9	Uncharacterized protein	CMBL	1	1	0.0159	-0.2989
F1S395	Uncharacterized protein	NQO1	1	1	0.0160	-0.3012
A0A287AJQ2	Phosphoglycerate mutase	PGAM1	1	1	0.0159	-0.3073
K9IVW4	Myotrophin	MTPN	1	1	0.0317	-0.3148
A0A287AR55	Fumarate hydratase, mitochondrial	FH	1	1	0.0159	-0.3185
A0A286ZKH3	Uncharacterized protein	SPR	1	1	0.0317	-0.3334
F1RMV5	ETHE1, persulphide dioxygenase	ETHE1	1	2	0.0465	-0.3443
Q6Y0X5	Sulfotransferase	SULT1A3	2	1	0.0195	-0.3444
Q1HFY1	Myosin regulatory light chain 2 protein	MYL7	2	1	0.0317	-0.3447
A0A286ZVQ0	Acyl-CoA thioesterase 4	ACOT4	1	1	0.0195	-0.3474
A0A287A0J0	Uncharacterized protein	MYL12A	1	1	0.0317	-0.3486
A0A286ZXI1	Glutaryl-CoA dehydrogenase, mitochondrial	GCDH	3	1	0.0317	-0.3508
I3LIE3	Myosin heavy chain 14	MYH14	1	2	0.0361	-0.3634
I3LLU0	Glycerol-3-phosphate dehydrogenase [NAD(+)]	GPD1L	1	1	0.0317	-0.3673
F1SUB1	Uncharacterized protein	PCBD1	1	1	0.0195	-0.3715
I3LJW4	Aconitase 1	ACO1	1	2	0.0365	-0.3757
Q9TUY2	Glyceraldehyde-3-phosphate dehydrogenase GAPDH (Fragment)	GAPDH	2	1	0.0317	-0.3793
A0A286ZLK7	Uncharacterized protein	ECI1	1	1	0.0459	-0.3843
A0A287ADK7	Mitochondrial fission 1 protein	FIS1	1	1	0.0365	-0.3888
A0A287AER8	Actin, alpha skeletal muscle	ACTA1	1	1	0.0491	-0.3922
B6VNT8	Cardiac muscle alpha actin 1	ACTC1	2	1	0.0317	-0.3923
C7AI81	Actin alpha 2	ACTA2	2	1	0.0317	-0.3923
A0A286ZWJ1	Uncharacterized protein	ACTG2	1	1	0.0317	-0.3964
F1SCD0	Uncharacterized protein	LOC396685	1	1	0.0361	-0.4157
I3L995	Uncharacterized protein	LRRC59	1	2	0.0159	-0.4218
A0A287AID2	Tropomyosin alpha-3 chain	TPM3	1	1	0.0317	-0.4274
A0A287B5T6	Uncharacterized protein	ARF3	3	1	0.0317	-0.4325
A0A286ZTI3	Uncharacterized protein	ARF1	1	1	0.0317	-0.4325
A0A287A217	Tubulin beta chain	TUBB4B	1	1	0.0195	-0.4465
F2Z5K5	Tubulin beta chain	TUBB4A	1	2	0.0268	-0.4479
A5YV76	Fatty acid synthase	FASN	1	1	0.0159	-0.4589
Q9GLW8	Peroxiredoxin 5	PRDX5	2	1	0.0317	-0.4600
A0A287BM57	Uncharacterized protein	ARF2	1	1	0.0159	-0.4609
F1SNL7	Uncharacterized protein	CNDP2	1	1	0.0317	-0.4678
A0A287BLV7	NADH:ubiquinone oxidoreductase core subunit S1	NDUFS1	1	1	0.0159	-0.4728
F1SDB7	Flavin-containing monooxygenase	FMO5	1	2	0.0159	-0.4733
F1SR80	Tubulin alpha chain	LOC100158003	3	2	0.0268	-0.4927
A0A286ZR94	Myosin-11	MYH11	1	1	0.0195	-0.5382

Table 4 (continued)

Accession number in SwissProt database	Protein description [*Sus scrofa*]	Gene Symbol	PE	SV	*p*-value	Log2 FC (SP + L *vs.* SP)
A0A286ZJD8	Dipeptidyl peptidase 7	DPP7	1	1	0.0481	-0.5451
F1S6M7	Tubulin beta chain	TUBB3	1	1	0.0159	-0.5924
A0A287BF56	Uncharacterized protein	ECHS1	1	1	0.0357	-0.6061
F1SUU6	Agmatinase	AGMAT	1	1	0.0159	-0.6554
Q56P20	ADP-ribosylation factor 4	ARF4	1	1	0.0159	-0.7094

PE Protein existence, *SV* Sequence version, *FC* Fold change, Dietary treatments, *SP* Spirulina diet, *SP + L* Spirulina diet supplemented with lysozyme

The table does not show the redundant proteins—to see all, please see Supplementary material—FileS3

impacting protein turnover and cellular homeostasis, similar to responses observed in intrauterine growth restriction piglets [55].

Spirulina supplementation appears to enhance energy conversion efficiency within the liver and increase functional capacity for detoxification and secretion at the cellular level. However, it is important to consider the possibility that some of the observed biomolecules may be products of stress or other adaptive responses that are not entirely beneficial to the liver. For example, the upregulation of stress-related proteins and metabolites could indicate a physiological effort to counterbalance potential oxidative stress or metabolic disturbances induced by Spirulina supplementation. While these processes may improve certain aspects of hepatic function, further investigation is needed to determine whether they also signal underlying strain on the liver that could have long-term consequences. Changes in stress response and cell mobility pathways suggest an improved liver response to dietary Spirulina inclusion, consistent with other studies [11, 57]. Spirulina may improve hepatic metabolic efficiency through alterations in fatty acid oxidation, carbohydrate catabolism and pyruvate metabolism, while also enhancing detoxification processes and cellular defence mechanisms. The modulation of nucleotide and steroid pathways indicates an impact on cellular function and hormonal signalling, with increased expression of actin filament-based movement and cellular response to stress factors observed in the SP group. It must be considered that all these affected pathways could also be related to the lower growth of the animals in the Spirulina group. To fully understand these associations, it would be interesting to correlate the metabolic findings herein obtained with microbiological and microalgae toxicity data.

Hepatic proteome—SP + L *vs.* Control: The effect of dietary Spirulina incorporation and lysozyme supplementation

In this comparison, the number of differentially expressed proteins was significantly lower than in the comparison discussed in the previous subchapter. This suggests that the inclusion of lysozyme in the Spirulina diet brought the physiological response of this group closer to that of the control animals. This observation is consistent with other parameters evaluated in our studies, such as the digestibility values of dry matter, organic matter and energy, where the SP + L group showed no significant differences compared to the control group [11].

In this comparison, all the differentially abundant proteins were more abundant in the SP + L group than in the control group.

Phosphoenolpyruvate carboxykinase, an enzyme involved in gluconeogenesis, was increased in the SP group. Elevated levels of this enzyme were observed in the liver of neonatal offspring of hyperglycaemic pig mothers, suggesting stimulated gluconeogenesis due to maternal hyperglycaemia [58]. An increase in this enzyme was also observed in a genetic diabetic pig model, suggesting a role for insulin in its expression [59]. The over-accumulation of this protein in the SP + L animals compared to the control group likely indicates a disturbance in energy metabolism due to Spirulina dietary inclusion, despite enzymatic supplementation. Understanding the activity of this enzyme could be beneficial in controlling blood glucose levels in piglets and understanding genetic predispositions to diabetes.

We observed an increase in CYP3A22, a cytochrome P450 enzyme involved in drug metabolism, which is significantly correlated with the biotransformation of midazolam in pigs [60]. Midazolam is used as a sedative and anxiolytic, and to induce anaesthesia in medical procedures, making it valuable in preclinical drug discovery [61]. Li et al. [62] measured the level of total cytochrome P450 to assess the impact of substances like enrofloxacin administered to Bama pigs. Howard et al. [63] highlighted the sensitivity of cytochrome P450 enzyme identification and quantification to variations in sample preparation methods, emphasizing the importance of reliable proteomic workflows. The increase in cytochrome P450 in

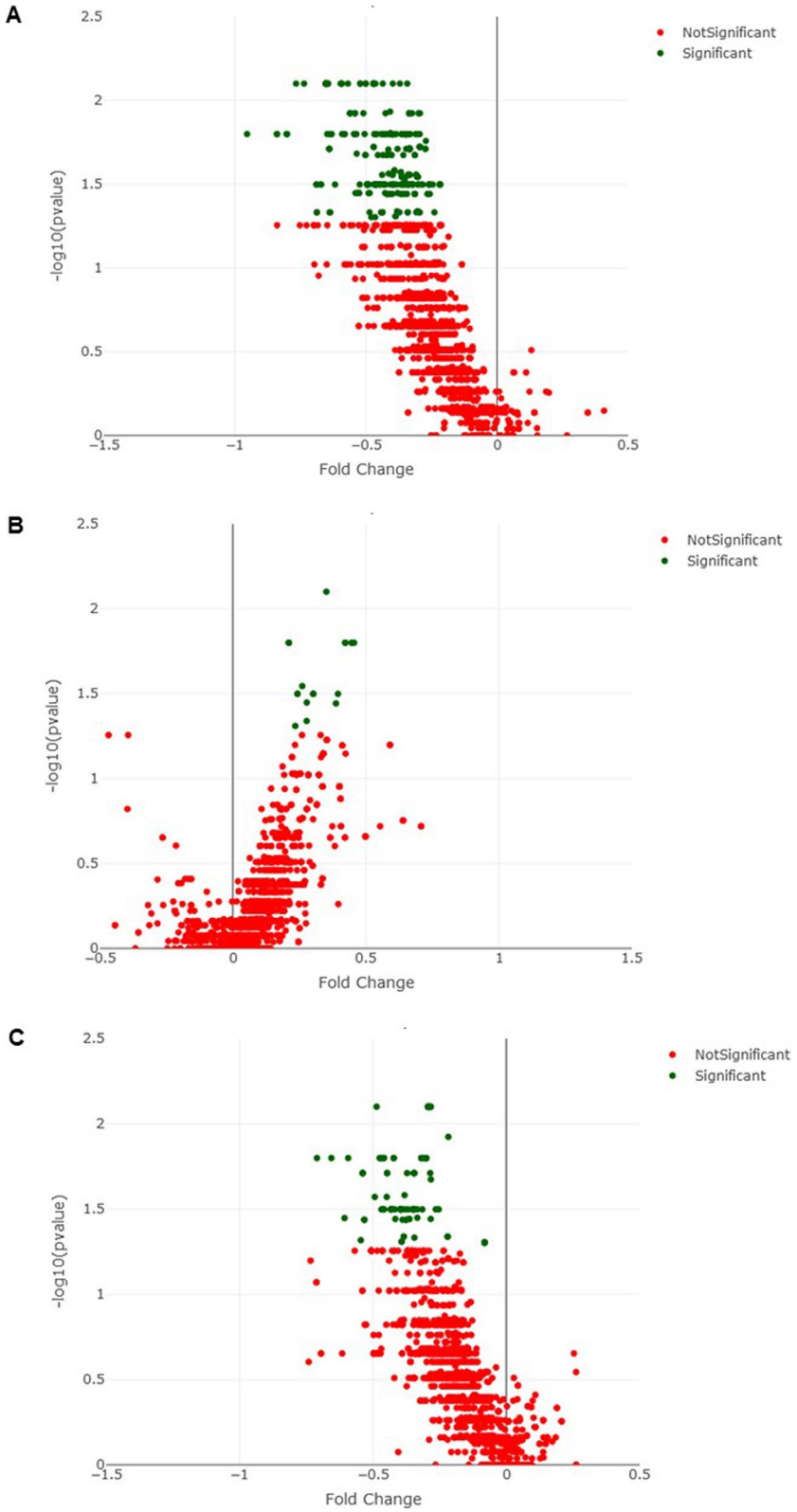


Fig. 8 Volcano plots for each comparison: SP *vs.* Control (**A**), SP + L *vs.* Control (**B**) and SP + L *vs.* SP (**C**). Significantly different proteins are marked in green, and non-significant are marked in red

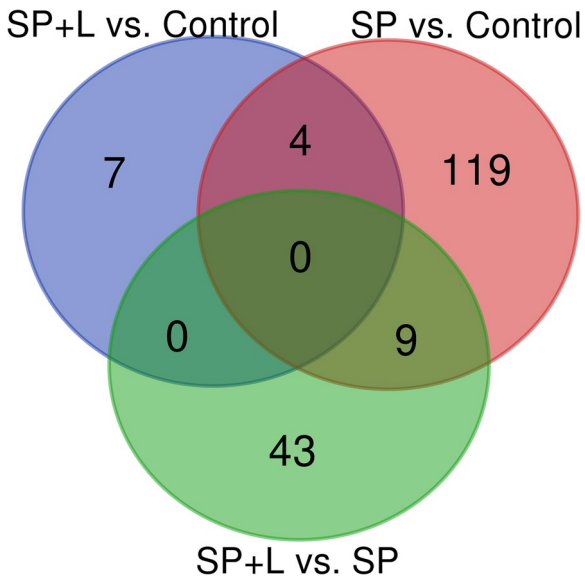


Fig. 9 Venn diagram showing the overlap between differentially expressed proteins in the three experimental groups

the SP + L group suggests a drug-like response in the liver to the dietary treatment.

Ghisaura et al. [64] found an increase in alpha-1 antitrypsin in *Sparus aurata L.*, hypothesizing a correlation with liver stress due to imbalances in feed composition. In our study, we observed an increase in alpha-1 antitrypsin in the SP + L group compared to control. Although both diets were balanced in composition, this correlation can be explained by the difficulty piglets might have in digesting the SP + L diet due to the presence of gelled proteins, as noted in our previous study [11].

Although only 11 proteins were differentially expressed between the two experimental groups in this comparison, they are associated with various biochemical processes. The effect of this treatment on carbohydrate metabolism at the liver level, through improved glucose regulation, appears to be an adaptive response to enhance energy availability. Additionally, there seemed to be a greater response in neutralising toxic substances in the liver, influenced by the observed effects on ammonium ions. The transport of essential proteins by the liver was also evidenced by the influence on transport by Golgi vesicles and the coating of COPII vesicles.

These findings highlight the intricate interplay between dietary components and liver function and underscore the potential of lysozyme supplementation to mitigate some of the adverse effects of Spirulina inclusion in piglet diets. Further research is needed to understand the underlying mechanisms fully and to optimise dietary formulations for improved animal health and performance.

Hepatic proteome—SP + L *vs*. SP: The effect of lysozyme supplementation on *Spirulina* diets

In this comparison, we noted that the number of differentially expressed proteins was greater than in the SP + L *vs*. control group comparison, with the SP + L *vs*. SP comparison showing 52 proteins. This suggests that lysozyme supplementation had a significant impact on the Spirulina diet, bringing the physiological response closer to that of the control group. This finding aligns with other parameters evaluated in our studies, such as digestibility values of dry matter, organic matter and energy, where the SP + L group showed no significant differences compared to the control [11].

All the differentially abundant proteins were more abundant in the SP + L group than in the SP group.

Aconitase, an enzyme involved in energy metabolism via glycolysis or the electron transport chain, is inactivated by superoxide and other oxidants [65]. Liu et al. [28] found a significant increase in this enzyme in the liver of new-born piglets after maternal folic acid supplementation. Although we did not determine the folic acid content of the diets in our study, it is inferred that the Spirulina diet was enriched in this vitamin, leading to such a response. The increase in aconitase in the SP + L group compared to the SP group may indicate greater availability of folic acid, possibly due to improved nutrient availability from lysozyme inclusion.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a crucial enzyme in the glycolytic pathway, playing a major role in energy metabolism. Martino et al. [66] highlighted its importance in the porcine liver proteome during hepatitis E virus infection. The increase in GAPDH in the SP + L group suggests higher levels of glycolysis, which could be a response to altered metabolic demands rather than pathogen exposure. While GAPDH is often associated with viral infections, due to its role in glycolytic and non-glycolytic pathways during viral replication, its upregulation in this experiment, where sanitary conditions were controlled and no pathogens were present, may indicate that the Spirulina and lysozyme supplementation stimulated metabolic processes linked to energy production and stress responses. This could be a result of increased cellular energy demands or oxidative stress rather than an immune response to a pathogen. Further studies are needed to elucidate whether dietary changes, such as Spirulina supplementation, could lead to metabolic shifts that mimic stress responses typically seen in pathogen-challenged animals.

The SP + L group exhibited higher levels of central lipogenic enzymes such as fatty acid synthase. Becker et al. [67] found that pigs fed a low-energy diet had higher levels of fatty acid synthase compared to those on a high-energy diet, indicating that higher dietary fat reduces

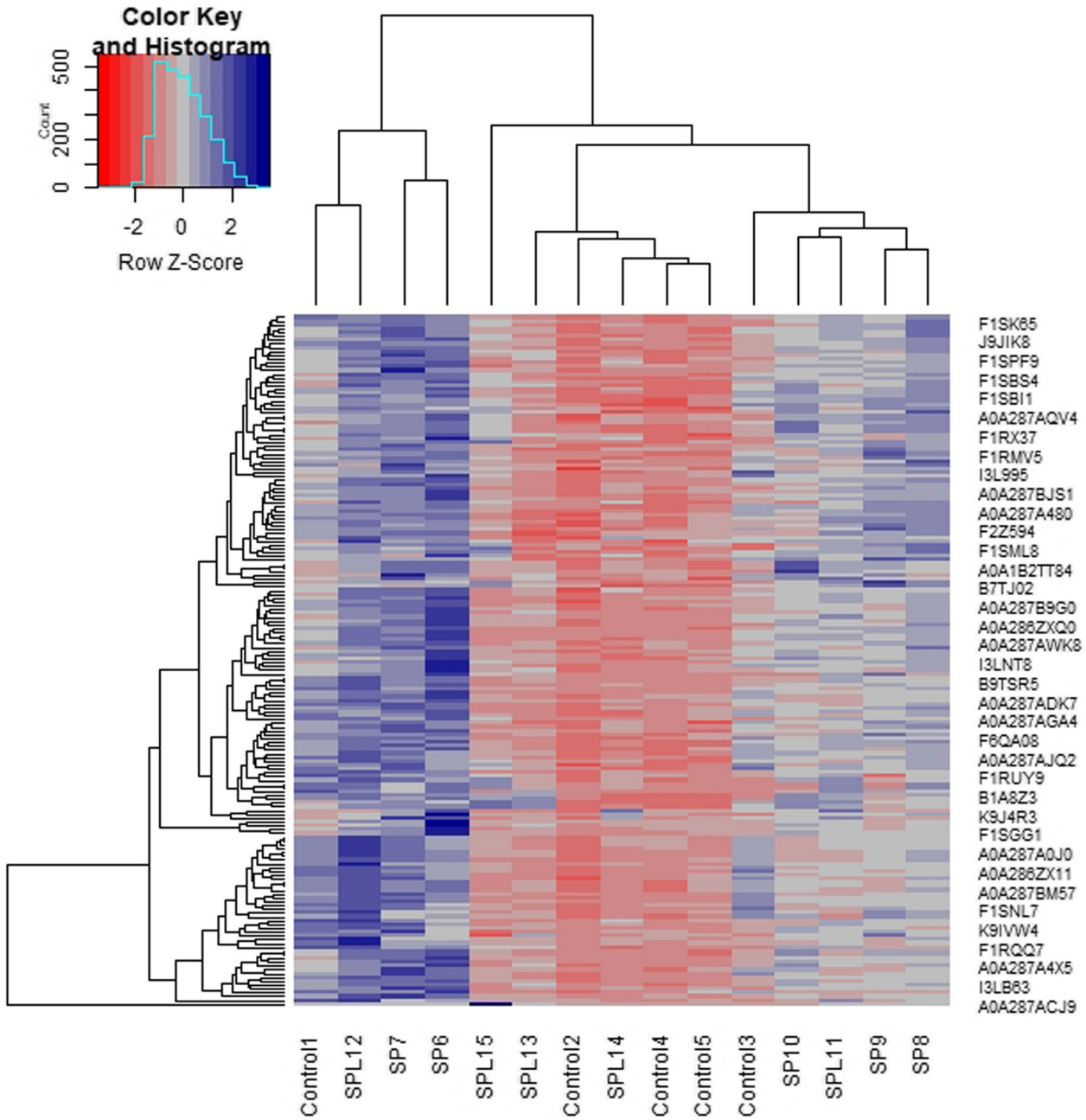
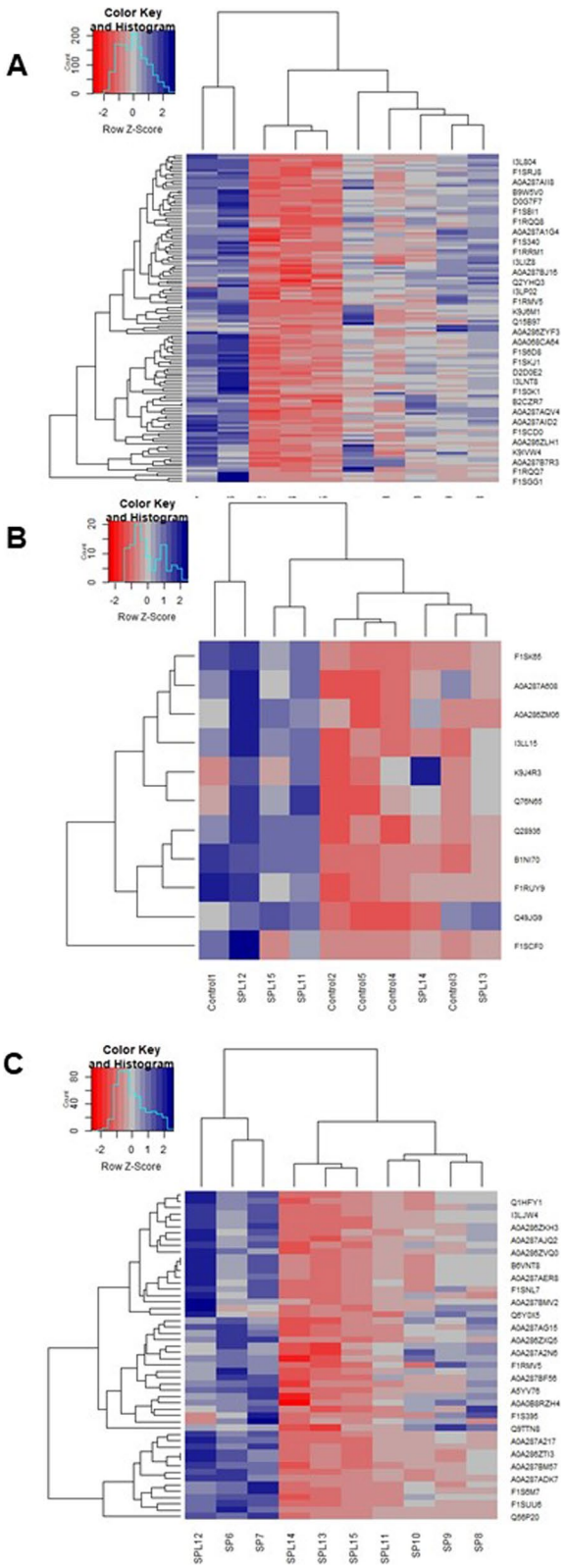


Fig. 10 Heatmaps of differentially abundant proteins in the piglet liver proteome considering all three experimental comparisons together. Protein accession numbers are displayed in rows and normalized protein abundance per sample is displayed in columns. Blue indicates abundance increases whereas red indicates abundance decreases. Control1, Control2, Control3, Control4, Control5 - Control diet; SP6, SP7, SP8, SP9, SP10 - 10% Spirulina diet; SPL11, SPL12, SPL13, SPL14, SPL15 - 10% Spirulina diet supplemented with lysozyme

fatty acid synthase and de novo lipogenesis. In our study, piglets fed SP + L had greater access to energy than the SP group, which is consistent with improved digestibility of energy [11]. This may justify the upregulation of central lipogenic enzymes, such as fatty acid synthase, which are involved in lipid biosynthesis. The increased expression of these enzymes suggests that lysozyme supplementation improved nutrient availability and energy utilization, leading to enhanced lipogenesis. This effect could be a response to the higher energy demands associated with the metabolic adjustments induced by Spirulina, promoting the synthesis of fatty acids to meet these demands.



◀ **Fig. 11** Heatmaps of differentially abundant proteins in the liver proteome of piglets fed different diets. Heatmaps showing protein intensity clusters for the SP+L *vs*. Control (**A**), the SP *vs*. Control (**B**) and the SP+L *vs*. SP (**C**). Protein accession numbers are displayed in rows and normalized protein abundance per sample is displayed in columns. Blue indicates abundance increases whereas red indicates abundance decreases. Control1, Control2, Control3, Control4, Control5 - Control diet; SP6, SP7, SP8, SP9, SP10 - 10% Spirulina diet; SPL11, SPL12, SPL13, SPL14, SPL15 - 10% Spirulina diet supplemented with lysozyme

Actin, specifically alpha skeletal muscle actin, was upregulated in the SP + L group. Wang et al. [55] found lower levels of this protein in the small intestine of intrauterine growth-restricted piglets, linking it to poor cytoskeletal structure and function. While our study found an upregulation of actin in the liver, it warrants further research as it contrasts with glycolytic observations. However, liver weight did not significantly differ between the groups, averaging 22.8 g/kg live weight ($P = 0.1331$, preliminary results, data not published). This finding suggests that, despite the observed changes in protein expression and metabolic pathways, there was no indication of hypertrophy or tissue damage in response to Spirulina or lysozyme supplementation. The lack of significant differences in liver weight indicates that the alterations in protein and metabolic processes were likely adaptive responses to the dietary treatments rather than signs of pathological changes in the liver. Therefore, the observed increases in proteins related to energy metabolism, such as GAPDH and fatty acid synthase, may reflect enhanced metabolic activity rather than a detrimental effect on liver structure or function. Spirulina with lysozyme supplementation positively influenced several crucial metabolic pathways, including energy metabolism, muscle function and cell development. A more specific analysis suggests that lysozyme supplementation improved metabolic efficiency by influencing the catabolic processes of small molecules. The muscular system was also affected, indicating that lysozyme benefits muscle development in piglets. This was also demonstrated in our previous study, where the SP + L group had a higher abundance of structural proteins and Krebs cycle activity compared to the SP group [13].

Improvements in coenzyme availability in the SP + L group suggest enhanced biochemical reaction efficiency, benefiting overall metabolism. Additionally, pathways involved in skeletal myofibril assembly and actin-mediated cell contraction, essential for muscle function, were increased. Lysozyme supplementation also positively impacted hepatic energy production mechanisms, increasing carboxylic acid catabolism and fatty acid oxidation pathways.

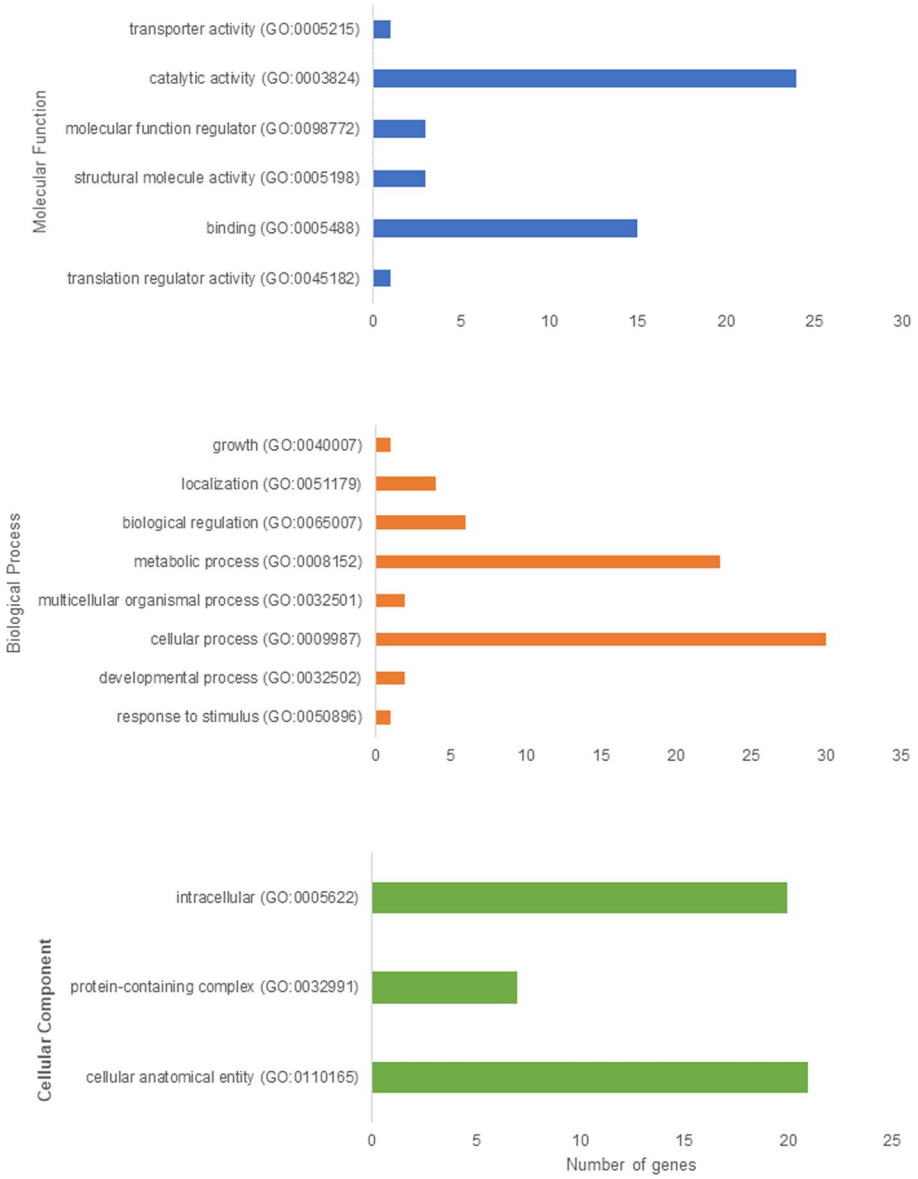


Fig. 12 Functional classification of differentially abundant proteins according to the molecular function, biological process and cellular component

Moreover, lysozyme supplementation promoted tissue regeneration or development processes by enhancing pathways associated with mesenchymal migration. The increase in the purine nucleoside biphosphate pathway may indicate a greater need for nucleic acid synthesis, potentially due to increased cell growth or division.

Conclusions

This study evaluated the effects of dietary Spirulina and lysozyme supplementation on piglet growth performance, hepatic metabolome and hepatic proteome. Spirulina inclusion impaired growth performance, as shown by a reduced final weight and an increased feed conversion ratio, while lysozyme did not mitigate these effects. Metabolomics analysis showed no significant differences in the liver metabolome among experimental groups, indicating primary metabolic processes remained largely unaffected.

Proteomic analysis revealed that Spirulina enhances energy conversion efficiency within the liver and increases the functional capacity for detoxification and secretion at the cellular level. It improves hepatic metabolic efficiency through alterations in fatty acid

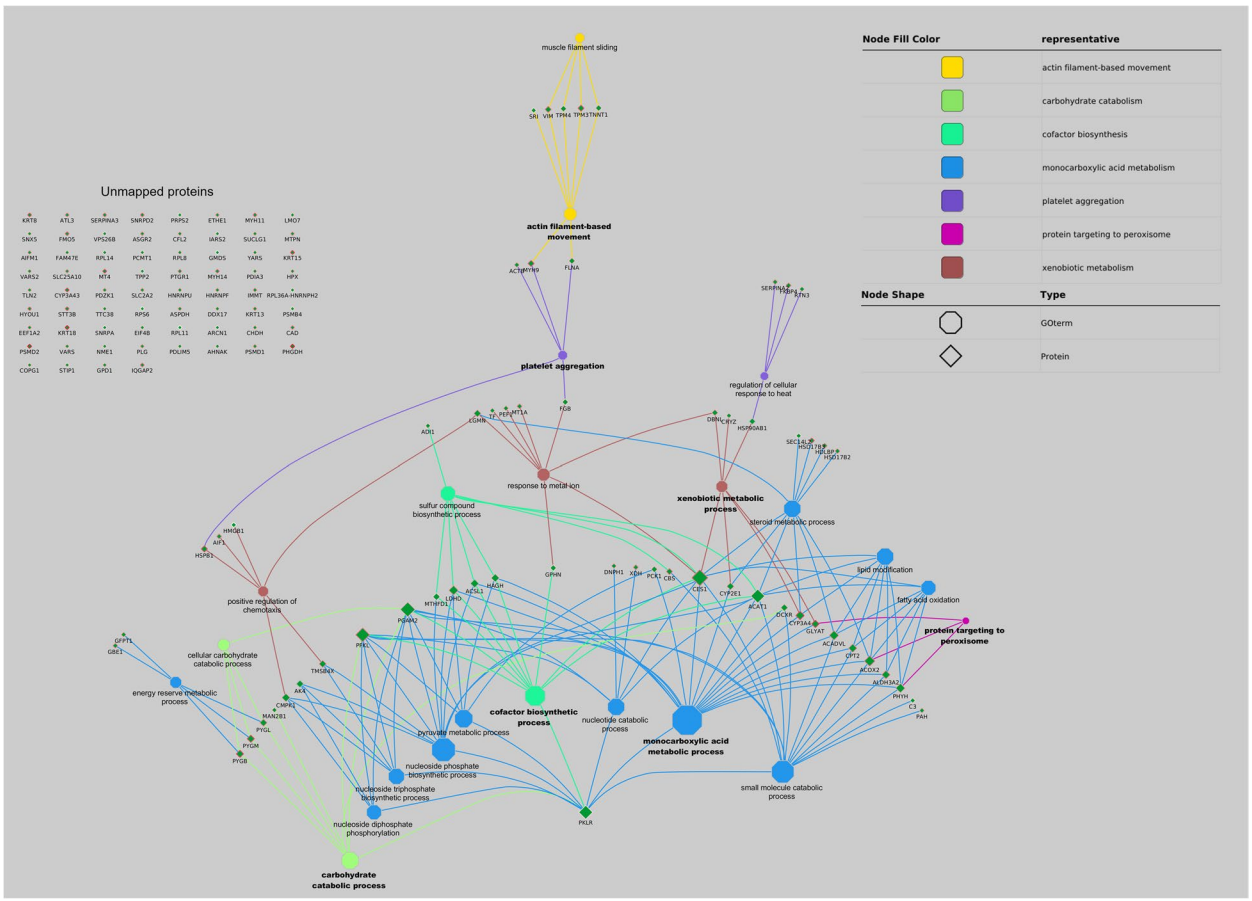


Fig. 13 Protein interaction network for the differentially expressed proteins in piglet liver in SP and Control groups comparison

oxidation, carbohydrate catabolism and pyruvate metabolism, while also enhancing detoxification processes and cellular defence mechanisms. The modulation of nucleotide and steroid pathways and increased expression of actin filament-based movement and stress response factors indicate significant impacts on cellular function and hormonal signalling. The metabolic pathways affected by the incorporation of Spirulina may also be associated with several adverse effects, including reduced growth performance and impaired nutrient digestibility. Specifically, the reduced final weight and increased feed conversion ratio observed in Spirulina-fed piglets highlight the potential negative impacts on growth efficiency. Additionally, the upregulation of stress-related proteins, such as heat shock proteins (HSPs) and metallothionein, suggests that Spirulina supplementation may induce physiological stress, potentially leading to metabolic strain on the liver. These findings indicate that while Spirulina offers some benefits in terms of metabolic efficiency, its inclusion in piglet diets may also trigger stress responses and reduce overall growth performance.

Furthermore, lysozyme supplementation shows potential in mitigating some adverse effects of Spirulina inclusion, bringing physiological responses closer to control levels. This was observed through decreased differentially expressed proteins and improved digestibility values of dry matter, organic matter, and energy. Improvements in coenzyme availability, skeletal myofibril assembly, and actin-mediated cell contraction suggest enhanced biochemical reaction efficiency and muscle function. Lysozyme also promoted tissue regeneration and development by enhancing pathways related to mesenchymal migration and nucleic acid synthesis.

The study underscores the potential of Spirulina as a dietary supplement but highlights the need to address its negative impact on growth performance. Lysozyme shows promise in mitigating adverse effects, suggesting a potential strategy for better dietary formulations. Future research should focus on optimizing Spirulina and lysozyme balance in diets, investigating specific metabolic pathways and assessing long-term impacts on animal health and productivity.

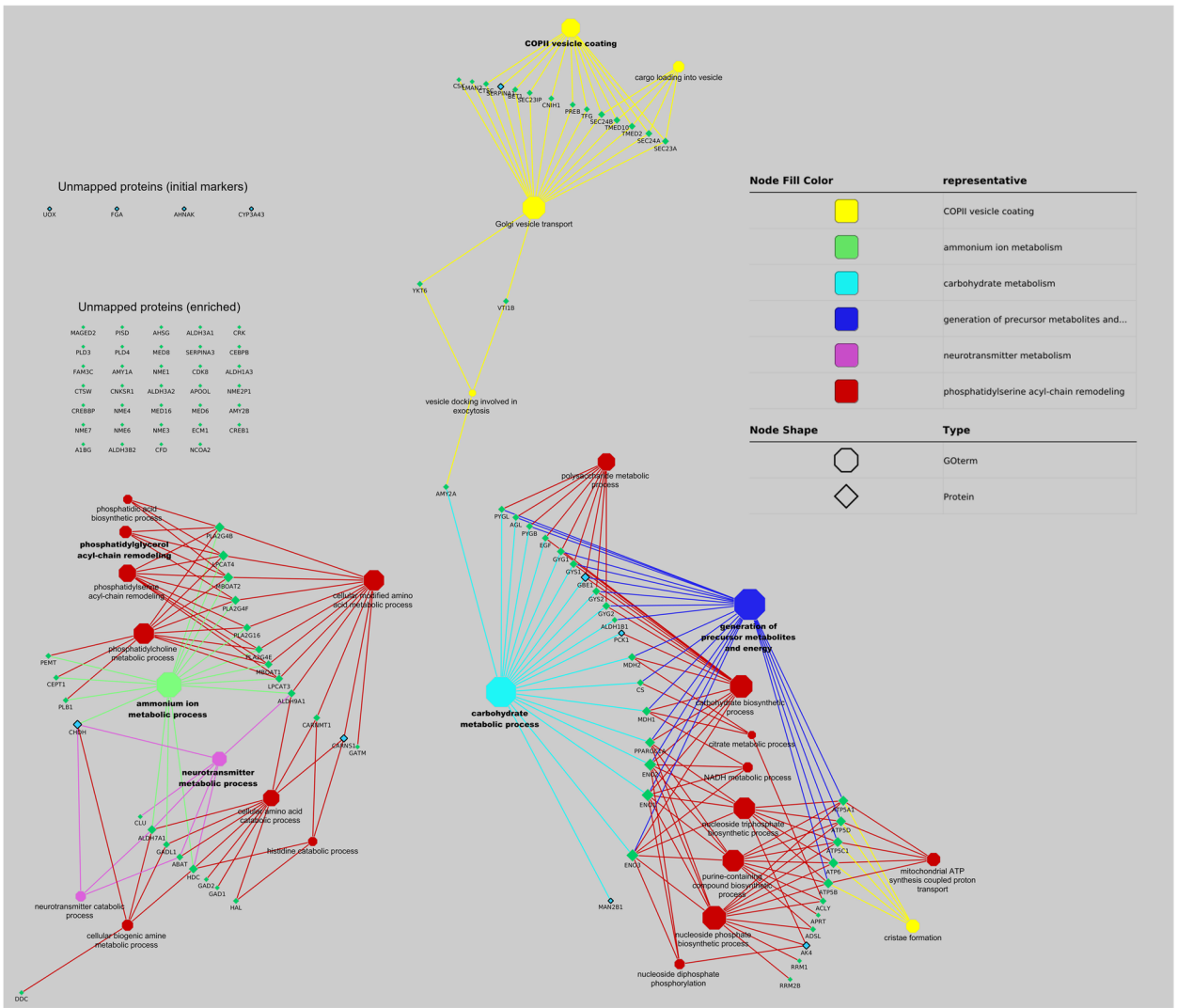


Fig. 14 Protein interaction network for the differentially expressed proteins in piglet liver in Control and SP + L groups comparison

Material and methods

Experimental design and sampling

The main details of animal trials and the composition of experimental diets used have been previously described [11]. Briefly, thirty post-weaned male piglets (Pietrain x (Large White × Landrace)) with an initial live weight of 11.9 ± 0.91 kg were purchased from a commercial farm (Valorgado, Montijo, Portugal). The animals were housed two by two in pens and had ad libitum access to feed and water. Each group of 10 piglets (5 pens) was fed with one of the three experimental diets: a cereal and soybean meal base diet (Control), a base diet with 10% Spirulina (SP) and an SP diet supplemented with 0.01% lysozyme (SP + L). The centesimal and proximal composition of diets is shown in Supplementary material—Table S2. After 4 weeks of the experimental period, animals were stunned using electrical stunning and slaughtered by exsanguination. The liver tissue of each animal was collected and stored at -80 °C until further analysis.

Metabolomic analysis—Sample preparation and NMR data acquisition

To prepare samples for metabolomic analysis, frozen liver tissue was kept frozen in liquid nitrogen and ground thoroughly. The extraction was performed using the chloroform/methanol method. Specifically, 300 mg of ground tissue was mixed with 480 µL of methanol, 480 µL of chloroform, and 250 µL of water. The homogenate was then centrifuged at 12,000x*g* for 10 min. The top fraction (methanol/water fraction) was carefully transferred into a new tube and vacuum-dried. The dried sample was re-suspended in 600 µL of phosphate buffer in D2O (80 mM; pH 7.4/pD 7.8; containing 107.0 µM of TSP-D4

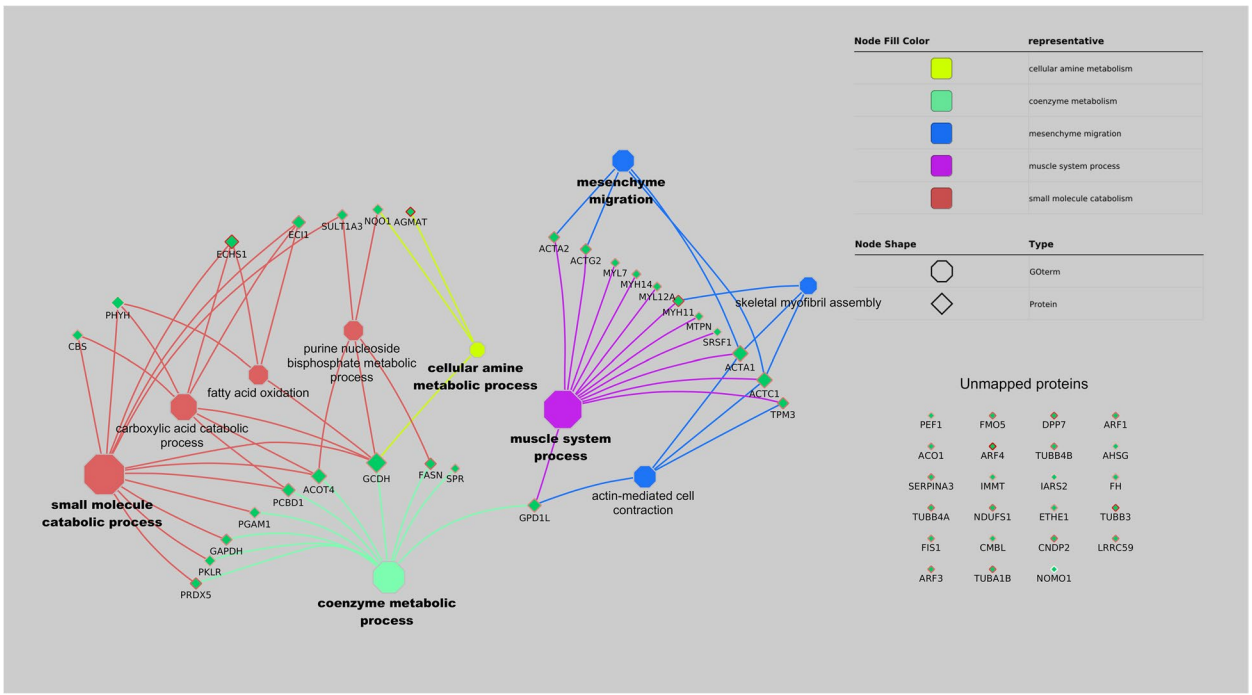


Fig. 15 Protein interaction network for the differentially expressed proteins in piglet liver in SP + L and SP groups comparison

(deuterated trimethylsilylpropanoic acid)) and centrifuged again at 12,000x*g* for 2 min. The supernatants were transferred into 5 mm NMR tubes rated for 800 MHz.

Proton (^{1}H) NMR spectroscopy was conducted using an 800 MHz Bruker AvanceII + spectrometer (Ettlingen, Germany) equipped with a room temperature triple resonance HCN Z-gradient probe at 298 K. Metabolite identification and quantification were performed using Chenomx NMR Suite 8.3 software (Chenomx Inc., Edmonton, Canada).

Proteomic analysis—Sample preparation for high-resolution LC–MS/MS and Tandem Mass Tag (TMT)

To perform protein extraction, liver tissue was homogenized with 500 mL of lysis buffer (100 mM TEAB, 2% SDS) using an Omni TH220 homogenizer (Omni International, Kennesaw, USA). The homogenate was subjected to two cycles of sonication at maximum amplitude (Qsonica, Newtown, USA) on ice. Following sonication, the sample was centrifuged at 16,000xg at 4 °C for 30 min, and the supernatant was transferred to a new tube. Total protein concentration was determined using a BCA assay (Thermo Scientific, Rockford, USA).

Subsequently, all samples were prepared using a filter-aided sample preparation (FASP) protocol and a Tandem Mass Tag (TMT)-based quantitative approach. Briefly, 35 µg of total protein diluted in urea buffer (8 M urea in 0.1 M Tris/HCl pH 8.5) were processed according to the FASP protocol with some modifications [68]. Samples were transferred to 10-kDa membrane filter units (Microcon YM-10, Merck Millipore, Burlington, MA, USA), centrifuged at 13,000xg for 20 min at 20 °C, and washed with 200 µL of urea buffer. Proteins were alkylated with 100 µL of iodoacetamide (50 mM IAA in urea buffer) for 20 min at room temperature in the dark. Following alkylation, the samples were washed twice with 100 µL of urea buffer and 100 µL of triethyl ammonium bicarbonate (100 mM, pH 8.5) (TEAB, Thermo Scientific, Rockford, USA), followed by centrifugation.

Proteins were digested overnight at 37 °C with 50 µL of 100 mM TEAB containing 2% trypsin gold (Promega Corporation, Madison, Wisconsin, USA) (enzyme-to-protein ratio 1:35, v/v). Post-digestion, the samples were centrifuged at 13,000xg for 10 min, washed with 50 µL of TEAB/ACN (1:1, v/v), and vacuum dried. The samples were then resuspended in 50 µL of 100 mM TEAB and labelled according to the TMT protocol [69]. Briefly, 19 µL of specific TMT label was added to each tryptic digest sample. After 60 min at room temperature, the reaction was quenched with 8 µL of 5% hydroxylamine (Sigma-Aldrich, St. Louis, MO, USA) and incubated for an additional 15 min. The TMT-labelled samples were randomly combined with an internal standard (labelled with TMT m/z 126), aliquoted, vacuum-dried, and stored at -20 °C until further analysis.

High-resolution LC–MS/MS analysis of TMT-labelled peptides was performed using an Ultimate 3000 RSLC-nano 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), loaded onto a trap column (C18 PepMap100, 5 µm, 100A, 300 µm × 5 mm), desalted for 12 min at a flow rate of 15 µL/min, and separated on an 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. This was followed by a gradient from 45 to 90% over 2 min, held at 90% for 2 min, and re-equilibrated at 5% B for 20 min at a flow rate of 300 nL/min. Mobile phase A consisted of 0.1% formic acid in water.

Ionization was achieved using a nanospray Flex ion source (Thermo Fisher Scientific, Bremen, Germany) with a 10 µm-inner diameter SilicaTip emitter (New Objective, Woburn, MA, USA). The mass spectrometer operated in positive ion mode using the data-dependent acquisition (DDA) Top8 method. Full scan MS spectra were acquired in the range from *m/z* 350.0 to *m/z* 1800.0 with a resolution of 70,000, 110 ms injection time, AGC target of 1×10^6, a ± 2.0 Da isolation window, and a dynamic exclusion of 30 s. Higher-energy collisional dissociation (HCD) fragmentation was performed at stepped collision energies (29% and 35% normalized collision energy) with a resolution of 17,500 and AGC target of 2×10^5. Precursor ions with unassigned charge states, as well as charge states of + 1 and > + 7, were excluded from fragmentation.

Data analysis

For metabolomics and proteomics analyses, liver samples from 10 and 5 animals per experimental group were used, respectively. Metabolomic data analysis was performed using the free online analytical platform MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/home.xhtml), following the methodology described by Yu et al. [70]. After uploading the data as a Comma-Separated Value (CSV) file, Pareto scaling was applied to normalize the data.

Proteomic data analysis involved the identification and quantification of proteins from acquired MS/MS spectra using the SEQUEST algorithm implemented in Proteome Discoverer (version 2.0, Thermo Fisher Scientific, Waltham, MA, USA). A database search was conducted against *Sus scrofa* FASTA files downloaded from the SwissProt database. The false discovery rate (FDR) for peptide identification was calculated using the Percolator algorithm in the Proteome Discoverer workflow, based on search results against a decoy database, and was set at 1%. Only proteins with at least two unique peptides and a 1% FDR were reported as confidently identified.

Protein quantification was achieved by correlating the relative intensities of reporter ions extracted from tandem mass spectra to those of the peptides selected for MS/MS fragmentation. An internal standard was used to compare relative quantification results for each protein between the experiments. The fold change between compared groups was calculated using the function log2 (Mean (group x) / Mean (group y)), as described by Yu et al. [70].

To categorize the proteins based on their molecular function, biological process, and cellular component, the list of identified proteins with significant differences between groups was screened against the *Sus scrofa* database using the PANTHER (Protein ANalysis THrough Evolutionary Relationships) online platform, Version 15.0 (release date February 14, 2020), available at http://www.pantherdb.org/.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-024-04339-7.

Supplementary Material 1.
Supplementary Material 2.
Supplementary Material 3.
Supplementary Material 4.
Supplementary Material 5.
Supplementary Material 6.
Supplementary Material 7.
Supplementary Material 8.
Supplementary Material 9.

Authors' contributions

J.P.B.F. and J.A.M.P. were responsible for the conception and experimental design. C.F.M., J.P.B.F., D.M.R. and A.M.A. performed the experimental part with animals. M.M. had performed the metabolomic analysis. J.K., A.H., N.G. and D.E. performed the proteomic analysis. C.F.M., A.M.A. and J.A.M.P. drafted the final paper, which was revised and approved by all members.

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Data availability

All data produced in this study are included in the published version. Datasets are accessible from the corresponding author on request.

Declarations

Ethics approval consent to participate

The experimental part with animals was carried out at the research facilities of the School of Agriculture (Instituto Superior de Agronomia- ISA), University of

Lisbon. The procedures were approved by the Ethics and Research in Animal Welfare Committee (ORBEA) of the ISA and approved (Authorization Number: 0421/2017) by the Animal Care Committee of the European Union legislation (2010/63/EU Directive).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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