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ORIGINAL ARTICLE

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Integrated metabolomics analysis of chill-stored rose shrimp (*Parapenaeus longirostris*) treated with different pressure levels of high hydrostatic pressure by ^{1}H-NMR spectroscopy

Qiuyu Lan[1] | **Ana Cristina De Aguiar Saldanha Pinheiro**[1] | **Giacomo Braschi**[1] | **Gianfranco Picone**[1] | **Pietro Rocculi**[1,2] | **Luca Laghi**[1,2]

[1]Department of Agricultural and Food Sciences, University of Bologna, Cesena, Italy

[2]Interdepartmental Centre for Industrial Agrofood Research, University of Bologna, Cesena, Italy

Correspondence
Luca Laghi, Department of Agricultural and Food Sciences, University of Bologna, P.za Goidanich 60, 47521, Cesena, Italy.
Email: l.laghi@unibo.it

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Abstract

The antimicrobial effects of high hydrostatic pressure (HHP) treatments on chill-stored seafood are well-documented, while their impact on the metabolic profile of seafood, especially the metabolome of fish flesh, and remains under-explored. Addressing this gap, this study investigates the effects of HHP on the metabolome of chill-stored rose shrimp by conducting multivariate data analysis based on untargeted proton nuclear magnetic resonance observations. Vacuum-packed rose shrimp samples were subjected to HHP at 0, 400, 500, and 600 MPa for 10 min and then stored at 2–4°C. The microorganism analysis and metabolic analysis were carried out on days 1 and 14. HHP treatment effectively deactivated *Lactobacillus* spp., *Escherichia coli*, *Pseudomonas* spp., total Coliforms, and sulfite-reducing anaerobic bacteria. Consequently, HHP treatment significantly reduced the formation rate of decay-related metabolites, such as hypoxanthine, trimethylamine, and biogenic amines, which exhibited significant accumulation in untreated samples. Multivariate unsupervised analyses provided insights into the overall changes in the metabolite profile induced by HHP. Metabolic pathway analysis revealed several pathways underlying spoilage, including pyruvate metabolism, valine, leucine, and isoleucine biosynthesis, purine metabolism, methane metabolism, glycine, serine, and threonine metabolism, citrate cycle (TCA cycle), glycolysis/gluconeogenesis, alanine, aspartate, and glutamate metabolism, sulfur metabolism, pantothenate and CoA biosynthesis, glutathione metabolism, and glyoxylate and dicarboxylate metabolism. Importantly, these pathways underwent alterations due to the application of HHP, particularly at high-pressure levels. In summary, the results unveil the potential mechanisms of HHP effects on chill-stored rose shrimps.

KEYWORDS
^{1}H-NMR, high hydrostatic pressure, metabolomics, pathway analysis, rose shrimp, seafood

1 | INTRODUCTION

Rose shrimp (*Parapenaeus longirostris*) is one of the most important commercial prawn species in the Mediterranean, with recorded landings increasing from 7000 tonnes in 1970 to 22,700 tonnes in 2021 (FAO, 2023). It is one of the most landed and highly consumed shrimp species in Greece, where it is consumed as unprocessed (Parlapani et al., 2020). However, rose shrimp is extremely susceptible to deterioration, even during iced storage, due to the rich content of protein, non-protein nitrogen compounds, and other nutrients that can allow microbial growth (Parlapani et al., 2020). Given the growing preference of consumers for fresh, healthy, and convenient seafood products, implementing a strategy to extend their shelf-life could increase their marketability.

High hydrostatic pressure (HHP) has been proven to be a reliable non-thermal food preservation method by inactivation of foodborne microorganisms and enzymes at low and ambient temperatures with minimal change in organoleptic properties and nutritional quality (Campus, 2011). This technology has been successfully applied to extend the shelf life of several species of shrimp and prawns, including black tiger shrimps (*Penaeus monodon*) (Kaur et al., 2015), white prawn (*Fenneropenaeus indicus*) (Ginson et al., 2015), and prawn (*Penaeus japonicus*) (Lopez-Caballero et al., 2000). HHP processing has the potential to reduce the initial microbial load, thereby enhancing the safety of seafood for consumers who prefer minimally cooked options.

Food metabolomics is a powerful analytical approach based on the comprehensive profiling of metabolites, providing broad insight into food quality, safety, and authenticity. It has been widely applied to various food matrices to evaluate the biochemical changes during processing and storage, thus contributing to the assessment of their quality (Ciampa et al., 2022; García-García et al., 2019; Huang et al., 2020). These studies highlight the potential of metabolomics to identify key metabolic markers associated with spoilage, freshness, and other quality attributes. Proton nuclear magnetic resonance (^{1}H-NMR) stands out as a valuable tool in the metabolites' profile evaluation step of food metabolomics, leveraging its intrinsic ability to rapidly and simultaneously quantify molecules with a wide array of functional groups. Notably, ^{1}H-NMR has proven effective in monitoring and determining metabolite alterations in seafood, as evidenced by previous studies (Ciampa et al., 2012; Deborde et al., 2021; Nair et al., 2016; Shumilina et al., 2015).

It should be noted, however, from the above literature review that limited studies are available on the effects of HHP on the metabolome of rose shrimps, and these effects remain largely unknown. To address this gap, our study aimed to observe and investigate the impact of HHP on the metabolome of rose shrimp during storage. We monitored changes in the metabolome of rose shrimps with and without HHP treatment (400, 500, and 600 MPa) after the first and 14 days using proton NMR-based metabolomic approaches. Coupled with multivariate data analysis, this allowed us to gain comprehensive insights into the metabolic changes induced by HHP treatments and subsequent storage. Overall, the information derived from this study could provide crucial insights into understanding the role and underlying mechanisms of metabolite alterations in cold-stored rose shrimp products treated with HHP.

2 | MATERIALS AND METHODS

2.1 | Sample preparation

Deep-water rose shrimps (*P. longirostris*) were harvested from the Adriatic Sea and fast frozen at −18°C for 24 h by the company Economia del Mare (Cesenatico, Italy). The samples were shell removed after thawing at a temperature of 4°C for 16 h. All of them were cut into pieces, divided into portions (15–20 g), and vacuum packed in a polypropylene (PP) tray containing six portions with a PP film for the subsequent analysis.

2.2 | HHP treatment

Vacuum packaged samples were pressurized in a 350 L chamber (HPP Italia s.r.l, Parma, Italy). Water was used as the pressure-transmitting medium. The samples were pressurized at 400, 500, and 600 MPa for 10 min at ambient temperature. Untreated samples were prepared as the control group. For each treatment, six packages were prepared for subsequent analysis.

2.3 | Storage

After HHP treatment, all samples were stored at 2 ± 1°C and underwent analytical assessments on days 1 and 14. For each HHP treatment, three portions were allocated for metabolomics analysis, and an additional three portions were designated for microbiological analysis on both day 1 and day 14. Each portion was derived from distinct packages.

2.4 | ^{1}H-NMR analysis

At each sampling time and HHP treatment, trichloroacetic acid extraction was conducted on three fish samples, with modifications to the procedure outlined by Ciampa et al. (2012). Specifically, 0.5 g of fish muscle was homogenized in 3 mL of 7% (w/w) trichloroacetic acid using an Ultra-Turrax (8630 g, 20 s). A 1 mL aliquot of the homogenate was then centrifuged at 14,000 rpm for 10 min at 4°C, and 0.7 mL of the supernatant was combined with 0.100 mL of 10 mM 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt (TSP) as a chemical-shift reference (δ, −0.017). Subsequently, the pH was adjusted to 7.0 ± 0.02 using 9 M KOH in an Eppendorf microfuge tube. After centrifugation (18,630 g, 10 min, at 4°C), 0.65 mL of the supernatant was transferred to an NMR tube for analysis.

To record ^{1}H-NMR spectra, an AVANCE III spectrometer (Bruker, Milan, Italy) operating at a frequency of 600.13 MHz was utilized, and it was equipped with the Topspin software (ver. 3.5). The ^{1}H-NMR spectra were acquired at 298 K using a CPMG pulse sequence with suppression of the solvent signal. The following parameters were set: the size of fid, 32 k; number of scans, 16; number of dummy scans, 16; spectral width, 12 pp;, acquisition time, 2.28 s; and delay d1, 5 s. Pre-processing of NMR spectra was performed in Topspin.

For calibration and quantification of the spectrum, signal assignment to compounds was carried out using Chenomx software (ver 8.3) by comparing with databases from Chenomx (ver. 10) and HMDB (release 2). Absolute quantification of molecules was conducted in a reference sample by adding 100 µL of 9.26 mM maleic acid as an internal standard. Spectra from other samples were adjusted to the reference using probabilistic quotient normalization (PQN) (Dieterle et al., 2006) to account for differences in water content. The concentration of each molecule was calculated from the area of one of its signals, measured by global spectra deconvolution in MestReNova software (ver. 14.2.0-26,256) with a line broadening of 0.3 and baseline adjustment by the Whittaker Smoother procedure.

2.5 | Microbiological analysis

Microbiological analyses were conducted on both untreated and treated (400, 500, and 600 MPa) rose shrimp samples. Throughout the storage period, all samples, immediately after undergoing HHP treatments, were examined for the presence of *Salmonella* spp. and *Listeria monocytogenes* following the protocols outlined in EN ISO 6579-1:2017/A1:2020 and ISO 11,290–1:2017, respectively.

The microbial groups considered in this study encompassed total mesophilic bacteria (TMB), *Lactobacillus* spp., *Pseudomonas*, sulfite-reducing anaerobic bacteria, total Coliforms, *E. coli*, and coagulase-positive staphylococci. For analysis, 10 g of samples were serially diluted using physiological saline solution (0.9% NaCl), and appropriate inoculum was either included or spread onto various selective culture media. These included plate count agar-PCA (Oxoid-Thermofisher, Milan, Italy) for TMB, De Man, Rogosa, and Sharpe agar MRS (Oxoid-Thermofisher, Milan, Italy) supplemented with cycloheximide (0.2% p/v) for *Lactobacillus* spp., Selective Pseudomonas Agar Base-PAB (Oxoid-Thermofisher, Milan, Italy) for *Pseudomonas* spp., Reinforced Clostridial Agar-RCA (Oxoid-Thermofisher, Milan, Italy) for sulfite-reducing anaerobic bacteria, Violet Red Bile Agar-VRBA (Oxoid-Thermofisher, Milan, Italy) supplemented with 4-methylumbelliferyl-β-D-glucuronide (MUG, Oxoid-Thermofisher, Milan, Italy) for total Coliforms and *E. coli*, respectively, and Baird Parker agar (BP) for coagulase-positive Staphylococci. Incubation durations were set at 24/48 h at 30°C for *Pseudomonas* spp. (PA) and 37°C for Lactobacilli, sulfite-reducing anaerobic bacteria, total Coliforms, *E. coli*, and coagulase-positive staphylococci. Sulphite-reducing anaerobic bacteria were incubated under anaerobic conditions using a gas-generating kit (Oxoid-Thermofisher, Milan, Italy).

2.6 | Statistical analysis

The significance difference between groups was tested by the analysis of variance (ANOVA) and calculated by Tukey's test ($p < 0.05$). Statistical analysis was carried out with the software with SPSS 8.0 for Windows.

Metabolites were considered as differently expressed due to treatment and storage time based on fold-change (absolute value of |log2 fold change| > 1) and p-value ($p < 0.05$) from ANOVA analysis, performed in SPSS (ver. 8.0) for Windows, with Tukey's as post-hoc test. The mean of the three replicates for each treatment/time point was used as a base for the multivariate unsupervised

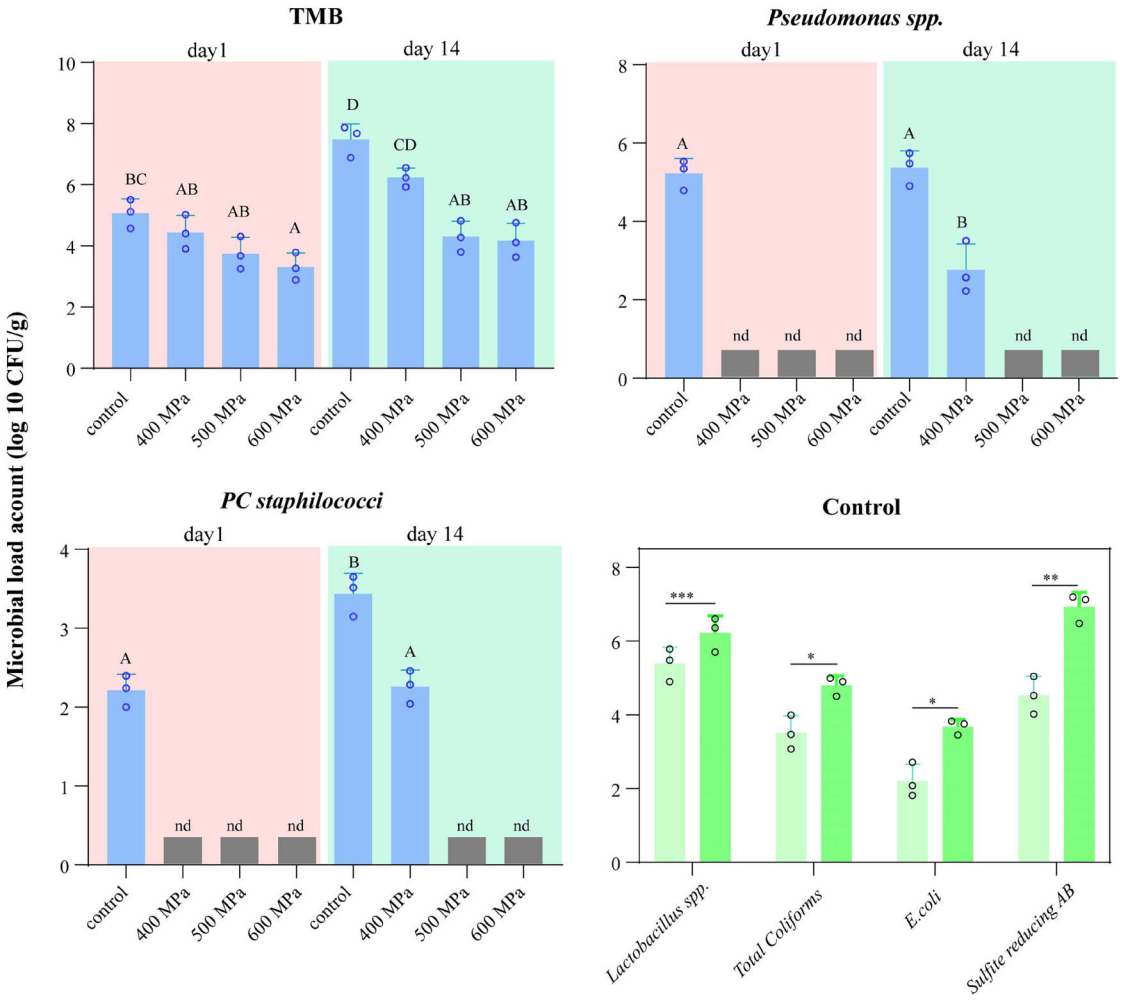

FIGURE 1 Changes in microbial cell loads ($\log_{10}$ CFU/g) of total mesophilic bacteria (TMB), *Pseudomonas spp*., and positive coagulase (PC) staphylococci at day 1 and day 14 of packaged rose shrimps in relation to the high hydrostatic pressure treatments applied (0, 400, 500, and 600 MPa). *Lactobacillus spp*., total Coliforms, *E. coli*, and sulfite reducing anaerobic bacteria (AB) of untreated rose shrimps at day 1 (light green) and day 14 (dark green). Different capital letters in the same row indicate significant differences ($p < 0.05$). *, **, ***indicates $p < 0.05$, 0.01, and 0.001, respectively.

data analyses hierarchical clustering analysis and robust principal component analysis (rPCA). The former was carried out by Origin Pro 2023 (OriginLab Corporation, Northampton, MA, USA), while the latter was carried out in R, by relying on the function PcaHubert.

Metabolic pathways mainly altered were highlighted using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) on the molecules evidenced by univariate analyses.

3 | RESULTS

3.1 | Effect of HHP on microorganisms

HHP treatment reduced the microbial load (Figure 1). In all samples, *Salmonella* spp., *L. monocytogenes*, and coagulase-positive staphylococci were never detected during the shelf-life of rose shrimps. Mean initial populations of total mesophilic bacteria (TMB), *Lactobacillus* spp., *Pseudomonas* spp., total Coliforms, sulfite reducing anaerobic bacteria (AB), *E. coli*, positive coagulase (PC) *Staphylococci* were 5.04, 5.34, 4.53, 5.17, 3.53, 2.27, and 2.20 lg CFU/g, respectively. 600 MPa treatments significantly ($p < 0.05$) reduced the initial TMB count by 1.7 lg cycles. The application of HHP treatments ranging between 400 and 600 MPa decreased the initial cell loads of *E. coli*, total coliforms, *Lactobacillus* spp., and sulfite-reducing AB compared to the untreated sample, under the detection limit (1 lg CFU/g).

After 14 days, untreated samples exhibited a significant ($p < 0.05$) increase in the counts of total mesophilic bacteria (TMB), *Lactobacillus* spp., total Coliforms, and sulfite-reducing anaerobic bacteria (AB), while there was no notable change in the count of *Pseudomonas* spp.

Following a 14-day storage period, in all treated samples, the counts of *Lactobacillus* spp., total coliforms, *E. coli*, and sulfate-reducing AB were all below the detection limit (1 lg CFU/g). *Pseudomonas* spp. and PC *Staphylococcus* were significantly influenced by the HHP levels, as both recovered under 400 MPa after 14 days. However, in samples treated at 500 and 600 MPa, counts of *Pseudomonas* spp. and PC *Staphylococcus* remained below their detection limits (1 lg CFU/g).

3.2 | Characterization of rose shrimp flesh metabolome

Representative ^{1}H-NMR spectra of rose shrimps under different pressures (0, 400, 500, and 600 MPa) on the first and last days of the present experiment are shown in Figure 1a. Multiple signals were recorded from 0.5 to 9 ppm, corresponding to various metabolites in rose shrimps. Sixty metabolites were unambiguously identified, comprising amines, amino acids and their derivatives, nucleotides and nucleosides, organic acids, carbohydrates, alcohols and polyols, and other metabolites. As depicted in the upset plot (Figure 2b), on day 1, identical metabolites were observed in both untreated and treated samples. However, after 14 days, tyramine and 3-methyl-2-oxovalerate were detected in untreated and 400 MPa-treated samples. Conversely, some metabolites (putrescine, cadaverine, and propionate) were exclusively detected in untreated samples, while other metabolites (IMP, L-α-glycerylphosphorylcholine, uridine, and 2-oxoglutarate) were solely undetected in the untreated samples.

To elucidate the main trend in the metabolites of rose shrimp due to different processing pressures on day 1 and day 14, their concentrations were expressed as distance matrix, then grouped utilizing hierarchical clustering. The heatmaps were generated, as shown in Figure 1c. Their visual inspection makes two main trends emerge. On day 1, the metabolites' profiles of untreated and treated rose shrimp samples were clustered together. When samples are observed after storage, the untreated samples on day 14 seem to stand out from any others, due to the extensive spoilage phenomena that will be described in deeper detail in the following sections.

3.3 | Multivariate analysis of rose shrimp's metabolome response to different pressure levels

To unveil the chemical distinctions between untreated and treated samples on both days 1 and 14, the dataset underwent rPCA. This analysis generated scores plots illustrating the grouping of classes and loadings highlighting the variables primarily influencing the observed clustering in the scores plot. The score plot is based on the first and second components (Figure 3a) and the bar plot for the correlation of metabolite concentrations with PC1 is shown in Figure 3b.

The samples collected on day 1 after treatments with 0, 400, 500, and 600 MPa appear indistinguishable, whereas those collected on day 14 show notable distinctions. PC1, capturing a substantial 94.4% of the total variance, predominantly governs the distribution of samples. Notably, HHP treatments emerge as the primary determinant, as evidenced by the samples collected on day 14 following a descending order along PC1 after being treated with 0, 400, 500, and 600 MPa. Intriguingly, the metabolites contributing to PC1 are linked to freshness. As depicted in Figure 3b, concentrations of glucose, pyruvate, IMP, trimethylamine N-oxide (TMAO), and certain amino acids display a high positive correlation with PC1. Conversely, metabolic byproducts such as acetate, ethanol, hypoxanthine, and TMAO, associated with seafood spoilage, exhibit a negative correlation with PC1.

3.4 | Comparison of differential metabolites in refrigerated rose shrimp under different stress levels of HHP

The differential metabolites were screened utilizing *p*-values and fold changes (FC) and shown in bubble plots (Figure 4a). To highlight the differential metabolites in chill-stored untreated samples, we compared the metabolite concentrations in untreated samples on day 1 and day 14. To identify the differential metabolites affected by HHP, we compared the metabolite concentrations in treated samples (at 400, 500, and 600 MPa) with those in untreated samples on both day 1 and day 14. We defined differential metabolites as those with a p-value < 0.05 and FCs > 2.0 or <0.5. The overlap of these differential metabolites is illustrated in the Venn diagram (Figure 4b).

In the untreated samples during storage, we identified 30 differential metabolites. Notably, the most substantial changes in all HHP-treated groups occurred on day 14 when compared to the control group. It is crucial to highlight that 22 of the differential metabolites in the control group exhibited significantly different changes with an opposite regulation after all applied HHP treatments. For example, metabolites such as trimethylamine (TMA), acetate, formate, uracil, tyramine, ethanol, succinate, hypoxanthine, propionate, cadaverine, and putrescine showed an upregulation in the untreated group. However, a comparison of metabolite contents with the untreated group indicated a down-regulation at day 14 after HHP

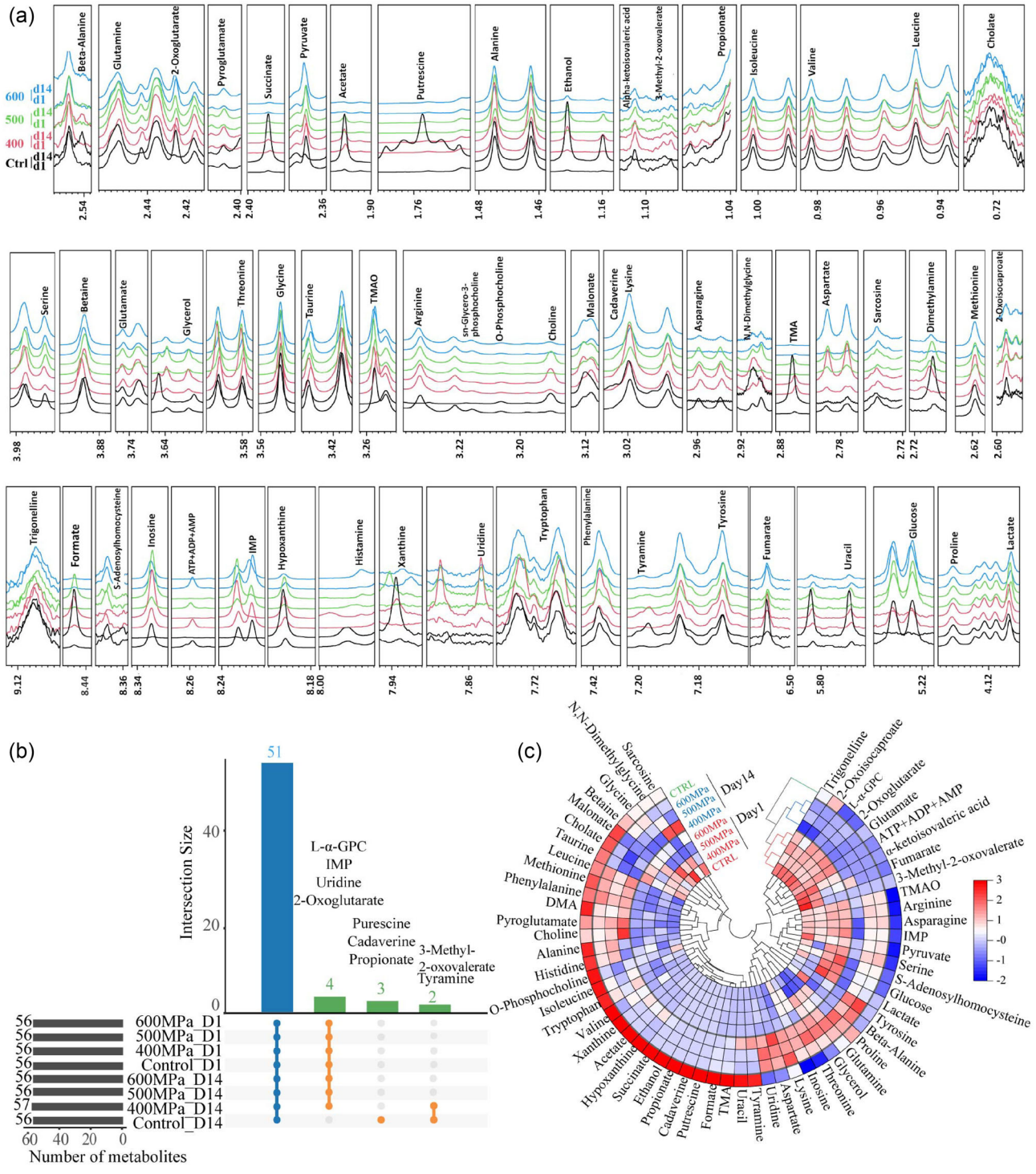

FIGURE 2 (a) Exemplary proton nuclear magnetic resonance (^{1}H-NMR) spectra recorded from the control and high hydrostatic pressure (HHP)-treated rose shrimp samples at day 1 (D1) and day 14 (D14). (b) Upset plot illustrating the distribution of metabolites in the control group and HHP-treated groups at day 1 and the day 14. (c) Heatmap presenting the variations in metabolites between the control group and the HHP-treated groups, collected at day 1 and day 14. TMA, trimethylamine; TMAO, trimethylamine *N*-oxide; L-α-GPC, L-α-glycerylphosphorylcholine.

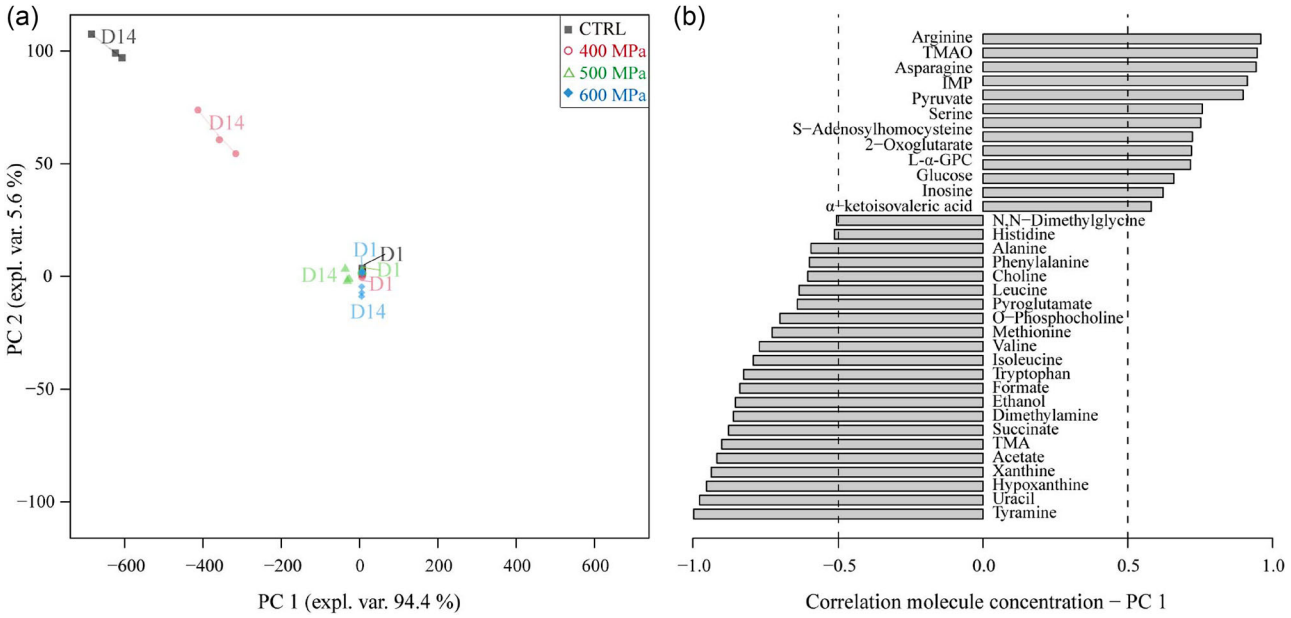

FIGURE 3 (a) Robust principal component analysis (rPCA) scores plot, and (b) rPCA loading plot of control group and high hydrostatic pressure (HHP; 400, 500, and 600 MPa) treated groups at day1 (D1) and day14 (D14). PC, positive coagulase; TMA, trimethylamine; TMAO, trimethylamine *N*-oxide; L-*α*-GPC, L-*α*-glycerylphosphorylcholine.

treatments. In contrast, metabolites like TMAO, pyruvate, tyrosine, serine, and inosine displayed a down-regulation in the untreated group but an up-regulation after all HHP treatments. Among these metabolites, the absolute fold change (FC) value of asparagine, acetate, hypoxanthine, xanthine, and uracil showed an increasing trend along with the pressure levels. Additionally, three differential metabolites in the untreated group (dimethylamine, choline, and pyroglutamate) did not exhibit significant changes (p-value < 0.05 and $|\log2(FCs)| > 1$) after any considered HHP treatments after 14 days. Tyramine and glucose were identified as differential metabolites in the untreated group, with significant changes (p-value < 0.05 and $|\log2(FCs)| > 1$) observed after high-pressure (500 and 600 MPa) HHP treatments. Apart from the differential metabolites screened in untreated samples, aspartate was characterized as a differential metabolite only in all treated groups.

3.5 | Key pathway analysis of chill-stored rose shrimp treated with different pressure levels of HHP

Pathway analysis was performed using the screened differential metabolites to identify the metabolic pathways during the storage of untreated rose shrimp (Figure 5a) and the metabolic pathways altered by HHP treatment (Figure 5b–d). These pathways with a p-value < 0.05 and path impact > 0 were considered significant.

In Figure 5a, we identified 12 significant pathways during the storage of untreated rose shrimp. In order of impact factors, these metabolic pathways include pyruvate metabolism, valine, leucine, and isoleucine biosynthesis, purine metabolism, methane metabolism, glycine, serine, and threonine metabolism, citrate cycle (TCA cycle), glycolysis/gluconeogenesis, alanine, aspartate, and glutamate metabolism, sulfur metabolism, pantothenate and CoA biosynthesis, glutathione metabolism, and glyoxylate and dicarboxylate metabolism. After HHP treatments, these pathways during the storage of untreated rose shrimp were mainly altered. In detail, nine key metabolic pathways were observed to change after undergoing 400 MPa HHP treatment except glutathione metabolism, purine metabolism, and valine, leucine, and isoleucine biosynthesis, followed by valine, leucine, and isoleucine biosynthesis affected after 500 MPa treatment, as well as purine metabolism, and valine, leucine, and isoleucine biosynthesis altered following 600 MPa treatment. Interestingly, all HHP treatments notably altered the alanine, aspartate, and glutamate metabolism with a high pathway impact and a lowest p-value.

Pathway analysis was conducted using the screened differential metabolites to identify metabolic pathways during the storage of untreated rose shrimp (Figure 5a) and those altered by HHP treatment (Figure 5b–d). These pathways, with a p-value < 0.05 and a path impact > 0, were considered significant.

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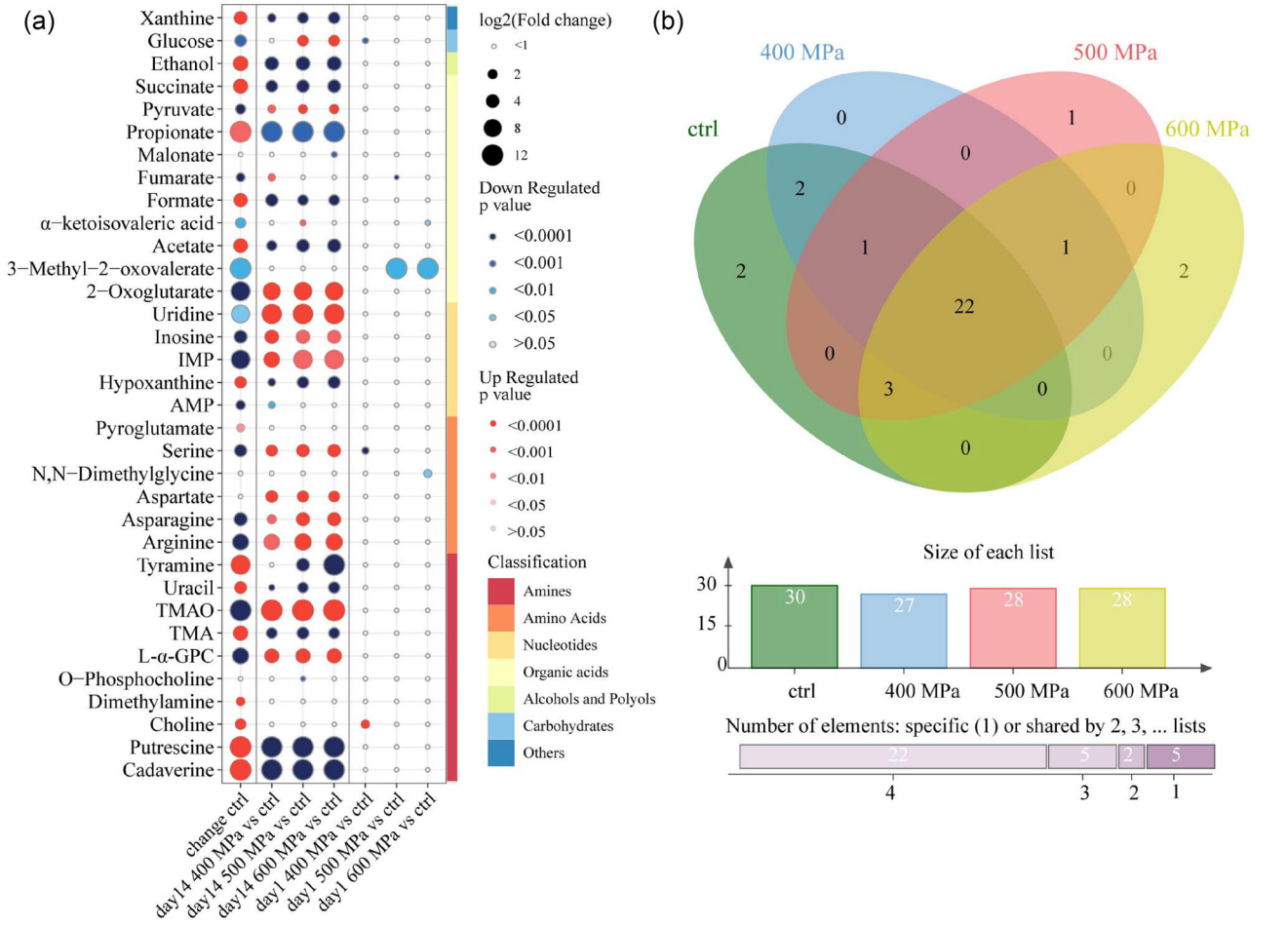

FIGURE 4 (a) Bubble plots exhibit the differential metabolites ($p < 0.05$ and fold changes [FC] > 2.0 or FC < 0.5). Differential metabolites of untreated group by comparing the metabolites levels of rose shrimp products at day 1 and those of untreated samples at day 14. Differential metabolites of treated samples by comparing the metabolites levels of treated samples and those of untreated samples at day 1 and at day 14. (b) Venn diagram showing the overlap of differential metabolites screened in bubble lots. TMA, trimethylamine; TMAO, trimethylamine N-oxide; L-α-GPC, L-α-glycerylphosphorylcholine.

pathways, ranked by impact factors, include pyruvate metabolism, valine, leucine, and isoleucine biosynthesis, purine metabolism, methane metabolism, glycine, serine, and TCA cycle, glycolysis/gluconeogenesis, alanine, aspartate, and glutamate metabolism, sulfur metabolism, pantothenate and CoA biosynthesis, glutathione metabolism, and glyoxylate and dicarboxylate.

Following HHP treatments, these pathways during the storage of untreated rose shrimp were significantly altered. Specifically, nine key metabolic pathways were observed to change after undergoing 400 MPa HHP treatment, excluding glutathione metabolism, purine metabolism, and valine, leucine, and isoleucine biosynthesis. Elevating the pressure to 500 MPa demonstrated a significant influence on the biosynthesis of valine, leucine, and isoleucine. Further increasing the pressure to 600 MPa resulted in a significant transformation of both valine, leucine, and isoleucine biosynthesis and purine metabolism. Interestingly, all HHP treatments notably affected the alanine, aspartate, and glutamate metabolism with a high pathway impact and the lowest *p*-value compared with the untreated samples.

3.6 | Potential mechanism of different pressure level treatments on chill-stored rose shrimps

The metabolic changes in grey mullets treated with HHP during chilled storage are illustrated in Figure 6, integrating insights from KEGG metabolic pathway analysis and prior studies (Chen et al., 2023; Wang et al., 2019). Four primary metabolic pathways are proposed: carbohydrate metabolism, proteolysis, energy metabolism, and nitrogen metabolism.

Glucose, the principal carbohydrate in rose shrimp and an energy source, enters the tricarboxylic acid cycle and pyruvate metabolism. This process leads to the

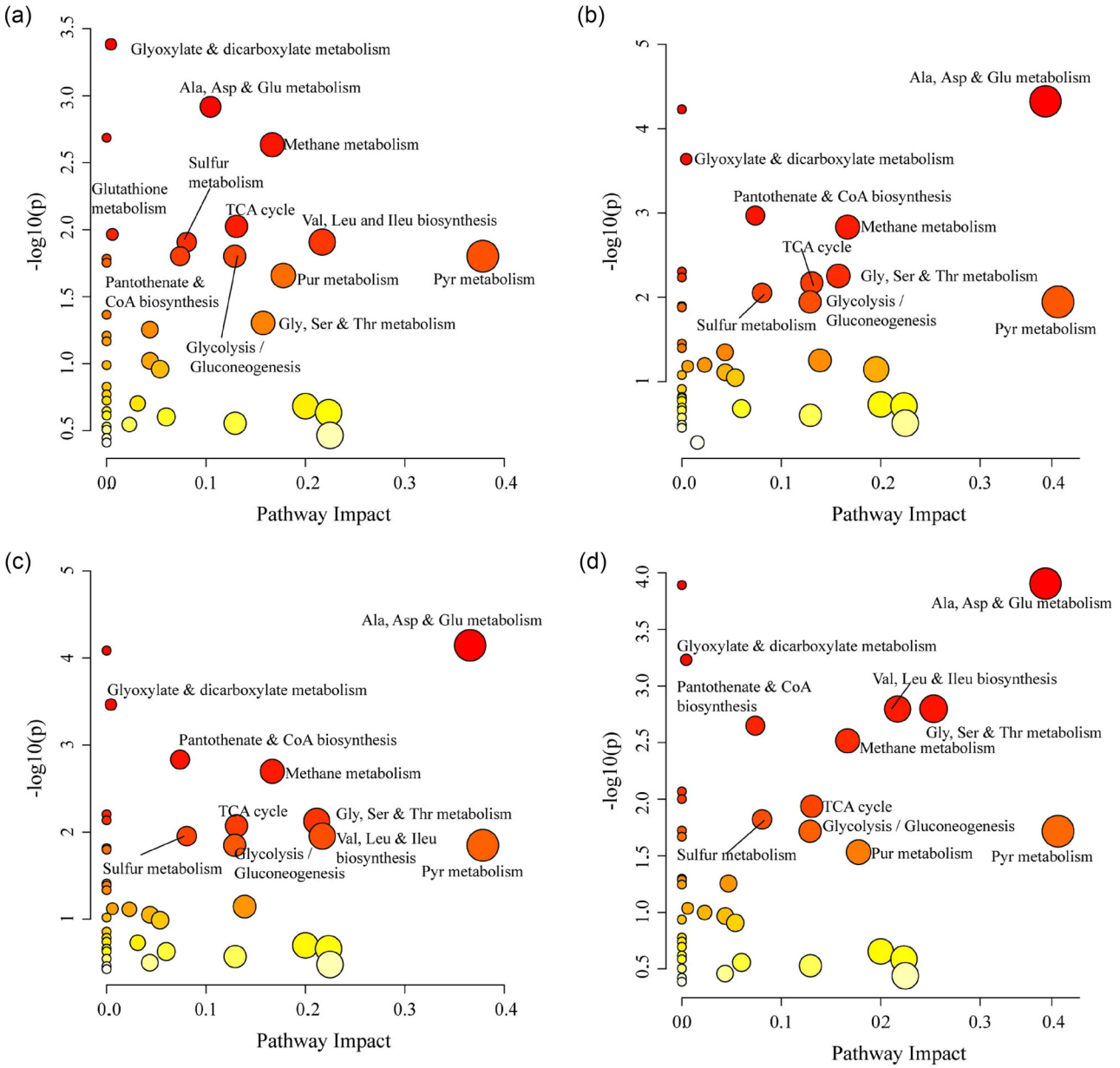

FIGURE 5 Pathway analysis for comparing the metabolic profiles of rose shrimp products (a) without treatment and the altered metabolites after applying (b) 400 MPa, (c) 500 MPa, (d) 600 MPa high hydrostatic pressure during storage.

accumulation of organic acids, notably succinate, acetate, and propionate, throughout the storage period, with a more pronounced accumulation in untreated samples. HHP treatment results in a deceleration of carbohydrate metabolite evolution.

Energy metabolism involves the autolytic decomposition of ATP, producing products such as ADP, AMP, IMP, inosine, and hypoxanthine, along with the degradation of uridine. In comparison with untreated rose shrimps, HHP treatment appears to slow down the degradation from IMP to hypoxanthine and the production of uracil from uridine after storage.

During the storage of untreated rose shrimp, various amino acids exhibit distinct concentration trends. Serine, arginine, and asparagine demonstrated a decreasing trend, while pyroglutamate and choline displayed an opposite trend after storage. HHP treatment could retard the degradation of serine, arginine, and asparagine, but it does not influence the production of pyroglutamate and choline. Biogenic amines, metabolic byproducts of amino acid catabolism, accumulate in untreated rose shrimps over the storage period. The primary spoilage-related biogenic amines in untreated rose shrimps are tyramine, putrescine, and cadaverine. It is noteworthy that all considered HHP

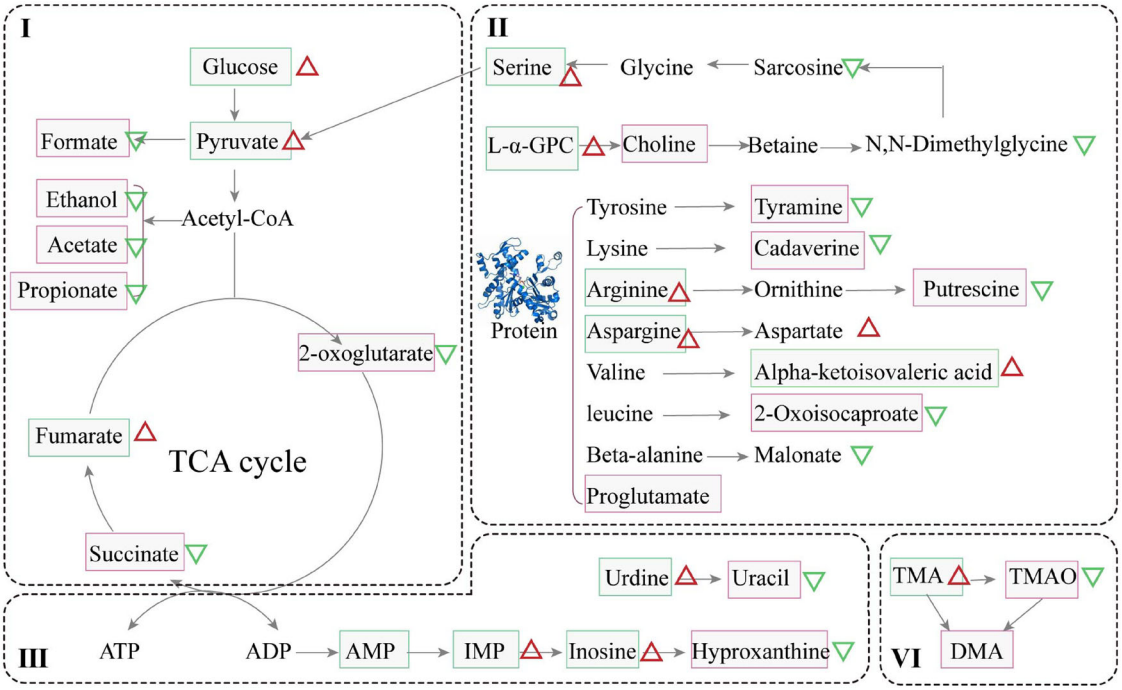

FIGURE 6 The potential metabolism network of differential metabolites. I, carbohydrate metabolism; II, proteolysis; III, energy metabolism; IV, trimethylamine metabolism. Metabolites in red square represent a significant increase during storage of untreated samples (fold changes [FC] > 2, $p < 0.05$), metabolites in green square represent a significant decrease during storage of untreated samples (FC < 0.5, $p < 0.05$). Red triangles represent a significant increase (FC > 2, $p < 0.05$) after high hydrostatic pressure (HHP) treatments, green triangles represent a significant decrease (FC < 0.5, $p < 0.05$) after HHP treatments. TMA, trimethylamine; TMAO, trimethylamine N-oxide.

treatments significantly inhibit the formation of putrescine and cadaverine. However, only high-level (500 and 600 MPa) HHP treatments significantly (p-value < 0.05 and |log2(FCs)| > 1) attenuate the biosynthesis of tyramine.

Nitrogen metabolism can contribute to the deterioration of fish freshness by generating TMA and dimethylamine. As illustrated in Figure 6, TMA and dimethylamine typically display an increasing trend in untreated samples during chilled storage. However, HHP treatments decelerated the accumulation of TMA compared to the control group, although no significant changes in dimethylamine were observed after HHP treatment.

4 | DISCUSSION

Shrimp is one of the seafood most vulnerable to rapid microbial growth due to its rich content of protein, non-protein nitrogen compounds, and other nutrients that can allow microbial growth (Don et al., 2018). HHP has been demonstrated effective in controlling various microbial groups responsible for seafood spoilage, including *E. coli*, *Pseudomonas* spp., *Staphylococcus aureus*, *Listeria* spp., *Enterobacteriaceae* spp., H_2S-producing bacteria, *Lactobacilli* spp., yeasts, and molds (Ekonomou et al., 2020; Lebow et al., 2017). This study demonstrates that HHP treatments significantly inactivate microorganisms in products based on rose shrimp, contributing to the preservation of their quality. Interestingly, after 14 days of storage, the counts of *Lactobacillus* spp., total coliforms, *E. coli*, and sulfate-reducing AB were all below the detection limit (1 log CFU/g) in all treated samples. However, *Pseudomonas* spp. and PC Staphylococcus recovered in samples treated with 400 MPa after 14 days. HHP treatments employed for bacterial inactivation are known to impact their metabolic profile. Studies by Kimura et al. (2018) indicated that 400 and 600 MPa HHP inactivate *E. coli* by affecting pathways related to central sugar metabolism and nucleic acid metabolism. The recovery of some bacteria during storage suggests that they can develop adaptive responses to HHP, which can be reflected in their metabolites' profiles. In line with this hypothesis, Chen et al. (2022) showed that adaptive responses of *Salmonella enterica* serovar Typhimurium to thyme and cinnamon oils resulted in changes in 47 metabolites, including lipids, oligopeptides, amino acids, nucleotide-related compounds, and organic acids. Understanding these metabolic adaptations would be a fruitful area for work aiming to optimize HHP treatments to ensure the inactivation of pathogens. In a previous study of ours, the application of high hydrostatic pressures inhibited microbial growth in striped prawn, thus reducing the

microorganisms' activities with direct effects on a wide range of molecules easily perceived by the consumer associated with freshness (Lan et al., 2022).

These intricate connections between microbial inactivation and metabolites' profiles suggest that the effects of HHP treatment on seafood could be fruitfully studied by combining microbial measurements with the quantification of the widest possible set of molecules. Metabolomics, the comprehensive analysis of small metabolites in biological samples, is perfectly tailored to understanding the biochemical responses and metabolic pathways associated with various treatments and ingredients (Utpott et al., 2022). By applying metabolomics, we can identify and quantify metabolites affected by HHP treatment that can clarify the mechanisms behind HHP preservation of chill-stored seafood.

In our study, we observed that significant changes in the metabolic profile of rose shrimp during storage mainly regarded organic acids, nucleotides, amino acids, and biogenic amines. These findings are consistent with previous studies on seafood post-mortem metabolic profiles. For instance, Guo et al. (2024) identified significant increases in organic acids, biogenic amines, and nucleotide degradation products during the post-mortem storage of shrimp. Similar changes in the metabolites' profile could also be observed in yellowfin tuna (*Thunnus albacares*) and salmon (*Salmo salar*) (Jääskeläinen et al., 2019). These changes are indicative of protein degradation, nucleotide breakdown, glycolysis, and nitrogen metabolism during the post-mortem storage of rose shrimp. In fact, in a previous study of ours, carbohydrate metabolism, nucleotide metabolism, amino acid metabolism, and nitrogen metabolism were involved in the spoilage metabolism pathway of chilled-stored grey mullet (Lan et al., 2024), similarly to what observed in this study. Moreover, Tan et al. (2023) reported that the mostly altered metabolic pathways during the post-mortem storage of sturgeon fillets were lipid metabolism, amino acid metabolism, and nucleotide metabolism.

Carbohydrate catabolism has been identified as a key metabolic process affected by seafood spoilage (Ma et al., 2020). Glucose, a primary energy substrate, is metabolized through the tricarboxylic acid cycle by spoilage microorganisms, driving deteriorative processes. The pyruvate metabolic process leads to the production of organic acids like succinate, acetate, and propionate, along with ethanol resulting from microbial growth in fish muscle (Prabhakar et al., 2020). In our study, the relatively higher levels of glucose in HHP treated rose shrimp might be attributed to the inhibition of both endogenous and bacterial enzymes by HHP, leading to its lower degradation (Zhao et al., 2020). This could have direct positive consequences for the maintenance of the freshness perceived by the consumer, as suggested by the lower levels of ethanol and organic acids. Intriguingly, the concentration of glucose exhibited a notable increase after 14 days with the increase in pressure levels. Conversely, one of the metabolic byproducts of glucose, acetate, displayed a decrease in concentration after 14 days under heightened pressure. The observed elevation in glucose levels and the simultaneous reduction in acetate levels after 14 days strongly indicate a discernible influence of pressure on glucose metabolism. This may signify a regulatory role for pressure in the underlying biochemical processes governing glucose utilization and its metabolic fate.

Another freshness-related pathway found in rose shrimp during storage is energy metabolism, which is the process of autolytic decomposition of ATP and the formation of its products (ADP, AMP, IMP, inosine, and hypoxanthine) (Greene et al., 1990). Among these nucleotides, IMP is one of the important compounds responsible for the umami taste of seafood, while inosine and hypoxanthine contribute to off-fish odor and bitterness (Yu et al., 2020). In our study, HHP treatment slowed down the degradation from IMP to hypoxanthine. This observation could be attributed to the inactivation of enzymes involved in the dephosphorylation of IMP to form inosine and hypoxanthine. It is notable to notice that the inhibitory potential of IMP degradation exhibits a pronounced dependence on applied pressure. With the elevation of pressure, there is a conspicuous deceleration in the pace of IMP degradation, resulting in the gradual formation of inosine and hypoxanthine. This phenomenon signifies a pressure-induced modulation of the enzymatic processes involved in IMP and inosine breakdown. Although the related enzyme activity was not determined in this study, evidence could be found in the research of Karim et al. (2019). They reported that HHP processing results in a significant reduction in 5′-NT and NP activity in haddock (*Melanogrammus aeglefinus*) and herring (*Clupea harengus*) fillets, with a decrease in enzyme activity observed with increasing pressure levels.

The accumulation of biogenic amines is associated with fish spoilage, since they are formed through microbial decarboxylation of the corresponding amino acids or by transamination of aldehydes and ketones by amino acid transaminases (Visciano et al., 2020). Thus, previous studies considered the overall content of biogenic amines or the content of single biogenic amines as a base for chemical indexes of freshness in different species of seafood (Arulkumar et al., 2023; Biji et al., 2016). In this study, tyramine, putrescine, and cadaverine were only detected after 14 days of chilled storage, while putrescine and cadaverine were only detected in untreated samples. The late accumulation of noticeable concentrations of these biogenic amines could be attributed to the low concentration

of their precursors in rose shrimp, relative to other species, with specific reference to tyrosine (Visciano et al., 2020). In addition, the observations on microorganisms suggest that the low accumulation of amines in treated samples is a direct consequence of the inactivation by HHP of biogenic amines-producing microorganisms, such as *Pseudomonas* spp., *E.coli*, and *Lactobacillus* spp., (Houicher et al., 2021; Wunderlichová et al., 2014). Additionally, there is evidence suggesting that HPP can inactivate the enzymes responsible for amino acid decarboxylation (Gokul Nath et al., 2023), further contributing to the reduction of biogenic amines.

TMA is produced by the decomposition of TMAO through nitrogen metabolism, mainly due to bacterial activity and is the main compound responsible for an unpleasant "fishy" odor. TMA content is often used as a biochemical index of spoilage in seafood. ^{1}H-NMR is proven as a quantitative method to quantify TMA content in seafood, with the advantage of being faster and more repeatable (Ciampa et al., 2022). In this study, all considered HHP treatments significantly slowed down the accumulation of TMA during chilled storage. It is worth noting that the impact of HHP treatment on TMA content in pressurized fish has been well documented in previous literature, albeit with varying results influenced by the matrix and applied procedures. Consistently with our results, Bindu et al. (2013) found TMA content of Indian white prawn (*Fenneropenaeus indicus*) was significantly reduced by HHP treatment at 100, 270, 435, and 600 MPa for 5 min at 25°C during chill storage. Interestingly, Erkan et al. (2011) found the TMA-N content in horse mackerel was not affected by HHP application at 220 MPa, 15°C for 10 min, 220 MPa, 25°C for 5 min, 250 MPa, 7−25°C for 10 min, 330 MPa, 3°C for 5 min, while HHP application at 330 MPa, 7°C for 10 min; 220−250−330 MPa, 25°C for 10 min significantly ($p < 0.05$) reduced TMA content. Despite the described peculiarities that can be noticed right after the HHP treatment, the main reduction in TMA concentrations can be undoubtedly noticed throughout storage, related to microbial activity.

5 | CONCLUSIONS

In our study, all considered HHP treatments effectively reduced the microbial load of rose shrimp products. Furthermore, metabolomic investigation unveiled the metabolic alterations in chill-stored rose shrimp induced by HHP treatment. Four major metabolic pathways were identified, namely amino acid, carbohydrate, energy, and nucleotide metabolism. Overall, HHP treatment had a notable impact on all these metabolic pathways, particularly influencing the production of key spoilage indicators such as TMA, tyramine, hypoxanthine, and ethanol. These findings provide valuable evidence on the role and underlying mechanisms of metabolite alterations in cold-stored rose shrimp products treated with HHP. While the inhibitory potential of nucleotide metabolism and some amino acid metabolism degradation exhibits a pronounced dependence on applied pressure. Comparisons with literature evidence that the effects of this non-thermal conservation treatment are deeply matrix-related, so further investigations of this nature should be extended to encompass various fish species to gain a comprehensive understanding of the treatment's applicability and efficacy.

AUTHOR CONTRIBUTIONS

Qiuyu Lan: Investigation; visualization; writing—review and editing; methodology; data curation; writing—original draft. **Ana Cristina De Aguiar Saldanha Pinheiro**: Conceptualization; writing—review and editing; data curation. **Giacomo Braschi**: Data curation; writing—review and editing. **Gianfranco Picone**: Writing—review and editing; data curation; visualization. **Pietro Rocculi**: Conceptualization; validation; methodology; supervision. **Luca Laghi**: Data curation; supervision; writing—review and editing; writing—original draft; methodology.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ORCID

Ana Cristina De Aguiar Saldanha Pinheiro https://orcid.org/0000-0001-8361-1728

Luca Laghi https://orcid.org/0000-0003-1593-7376

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