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Comparative metabolomic analysis of minced grey mullet (*Mugil cephalus*) pasteurized by high hydrostatic pressure (HHP) during chilled storage

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ABSTRACT

The beneficial antimicrobial effects of high hydrostatic pressures (HHP) treatments on chill-stored seafood are well documented. Less attention has been given to their impact on the metabolic profile of the seafood, which can offer valuable insights into the preservation mechanisms of these treatments. To address this gap, the present work describes the effects of HHP on the metabolome of chill-stored grey mullet using ^{1}H-NMR. Samples were treated with HHP at 0, 400, 500, and 600 MPa for 10 min and stored at 2–4 °C. HHP effectively inactivated *Lactobacillus* spp., *E. coli*, *Pseudomonas* spp., total Coliforms, and sulfite-reducing anaerobic bacteria (AB), and significantly ($p < 0.05$) reduced the total mesophilic bacteria (TMB). Additionally, the microbiological shelf-life of grey mullet was extended from 9 to 35 days with the application of 600 MPa HHP. Unsupervised hierarchical cluster analysis and robust Principal Component Analysis (rPCA) revealed the overall changes in metabolites' profile caused by HHP. HHP treatment significantly reduced the formation rate of decay-related metabolites, including hypoxanthine, trimethylamine (TMA), and biogenic amines, which significantly ($p < 0.05$) positively correlated with all considered microorganisms. A metabolic pathway analysis revealed several pathways that may underly the spoilage of grey mullet, including tyrosine metabolism, pyruvate metabolism, lysine metabolism, glycolysis/gluconeogenesis, butanoate metabolism, citrate cycle (TCA cycle), phenylalanine metabolism, and sulfur metabolism. Interestingly, among these pathways, lysine metabolism remained unaffected by the considered HHP treatments. These findings provide a better understanding of the mechanisms underlying the effects of HHP on chilled grey mullet.

1. Introduction

Flathead grey mullet (*Mugil cephalus*) is a seafood fish found worldwide, highly appreciated for human consumption in the Mediterranean region, Asia, and the United States (Donatella, 2015). Although its market price is lower than other premium seafood, grey mullet boasts unique flavor characteristics and a delicate texture, making it suitable for creating novel value-added products. To elevate its market appeal and meet consumer demand for convenient seafood products, one option is to use it as a base for minced ready-to-eat uncooked products. This innovative preparation preserves the unique flavors and delicate texture of grey mullet while providing a convenient option that aligns with modern culinary preferences.

Unfortunately, grey mullet products are extremely perishable during chilled storage due to chemical reactions, enzymes (both endogenous or exogenous), and the activity of microorganisms, particularly Gram-negative and psychrotrophic (Ghaly et al., 2010; Parlapani et al., 2021). The consequent short shelf life presents challenges for storage and distribution. To extend it, non-thermal treatments based on high hydrostatic pressure (HHP) are attractive options. HHP has been recognized to inhibit microbial growth and enzymatic activity without affecting the fish's metabolites' profile as deeply as thermal processing, thereby preserving the quality and freshness of the product (Cheftel & Culioli, 1997). An example of their application can be found in the works of Erkan and Üretener (2010) and Senturk and Alpas (2012), who applied pressures of 200–400 MPa for 5–15 min at 3–25 °C to improve the shelf life and sensory values of red mullet (*Mullus surmelutus*) and Atlantic Mackerel (*Scomber scombrus*), respectively. The impact of HHP treatments on seafood preservation varies widely, influenced by process parameters and by the specific characteristics of the seafood species. A piece of evidence of such variability comes from recent observations on

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Abbreviations

3-HDIV	3-Hydroxyisovalerate
O-AC	O-Acetylcholine
TMA	Trimethylamine
TMAO	Trimethylamine N-oxide
L-α-GPC	sn-glycero-3-phosphocholine
PSC	O-Phosphocholine
5-AP	5-Aminopentanoat
DMG	N,N-Dimethylglycine

trimethylamine (TMA). Kaur et al. (2013) found no significant changes in TMA levels of black tiger shrimp (*Penaeus monodon*) after HHP treatments (100, 270, and 435 MPa for 5 min), while Lan et al. (2022) found that the application of HHP at 400 MPa for 10 min increased the TMA concentration to 0.70 mg/100 g in striped Prawn (*Melicertus kerathurus*), compared to 0.48 of the control. However, studies specifically examining the impact of HHP on grey mullet products are comparatively limited.

To gain a comprehensive understanding of the effect of HHP on grey mullets, we can rely on metabolomics, based on the comprehensive analysis of small molecular metabolites in biological samples. Metabolomics enables us to identify and quantify specific metabolites affected by HHP treatments, providing a better understanding of the underlying biochemical alterations and metabolic pathways involved. Proton nuclear magnetic resonance (^{1}H-NMR) can be a convenient tool for this purpose, as its physical principles enable it to be intrinsically quantitative, regardless of the chemistry of the molecules observed. Examples of the application of this platform to study seafood decay along storage can be traced in the works by Ciampa et al. (2022) and Shumlina et al. (2015).

From the above literature review, however, it is evident that limited studies exist on the effect of HHP on the metabolome of grey mullet, which has motivated the present study. Therefore, this investigation aims to comprehensively elucidate the metabolic alterations induced by HHP treatments and subsequent storage in grey mullet flesh, while also examining their correlation with microorganisms. To achieve this, the metabolite profiles of grey mullet flesh subjected to varying HHP intensities (0, 400, 500, and 600 MPa) are analyzed using proton NMR-based metabolomic techniques, alongside microbial analysis. Additionally, the study employs multivariate data analysis and correlation analysis to elucidate the relationship between the metabolome and microorganisms. Overall, the information derived from this study could provide insights into the role and underlying mechanism of metabolic alterations in cold-stored grey mullet products treated with HHP.

2. Materials and methods

2.1. Sample preparation

Grey mullets (*Mugil cephalus*) used in this study were fished on March 2019 in the Adriatic Sea (42°30′N, 13°30′E). They were fast frozen at −18 °C for 24 h by the company Economia del Mare (Cesenatico, Italy). After thawing (at 4 °C for 16 h), fishes were skinned and manually cut into pieces and then divided into 6 portions (15–20 g), vacuum packed in polyethylene terephthalate (PET) trays with PET film.

2.2. HHP treatment

HHP treatments were performed in a laboratory-scale unit with 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, 21 packages were prepared.

2.3. Storage

After the HHP treatments, all samples were stored at 2 ± 1 °C and subjected to each analytical determination after 1, 6, 9, 14, 21, 28 and 35 days. Each sample's shelf life was determined based on the point at which it reached a microbial load of 6 log10 CFU/g (de Aguiar Saldanha Pinheiro et al., 2023). For each treatment and storage time, 3 portions were used for metabolomics analysis, stored at −80 °C, and 3 portions were used for microbiological analysis. Each portion was from a different tray.

2.4. Microbiological analysis

Microbiological analyses were performed on untreated and treated (400, 500, and 600 MPa) grey mullet samples according to de Aguiar Saldanha Pinheiro et al. (2023). All samples, immediately after HHP treatments and throughout the storage period, were subjected to analysis for the presence of *Salmonella* spp. and *Listeria monocytogenes* following the standard ISO detection protocols 579–1:2017/A1:2020 and ISO 11290–1:2017, respectively. Subsequently, for each sample, five suspect colonies (or all colonies if fewer than five were present) were isolated and subjected to identification through the biochemical tests outlined in each ISO protocol.

Microbial groups considered in this research were total mesophilic bacteria (TMB; ISO 4833–1:2013), *Lactobacillus* spp. (ISO 15214), *Pseudomonas* spp. (ISO 13720:2010), sulfite-reducing anaerobic bacteria (ISO 15213:2003), total Coliforms, and *E. coli* (ISO 16649–2:2018). The microbial groups were determined according to the corresponding ISO protocols. Briefly, 10 g of each sample was serially diluted using a physiological saline solution (0.9% NaCl) and a portion of such diluted material was distributed in different selective culture media (Oxoid-Thermofisher, Milan, Italy). These include plate count agar (PCA) for TMB; De Man, Rogosa, and Sharpe agar MRS supplemented with cycloheximide (0.2% p/v) for *Lactobacillus* spp.; selective *Pseudomonas* Agar Base-PAB for *Pseudomonas* spp.; Reinforced Clostridial Agar-RCA for sulfite-reducing anaerobic bacteria; Violet Red Bile Agar-VRBA supplemented with 4-Methylumbelliferyl-β-D-glucuronide (MUG) for total Coliforms and *E.coli*, respectively. Plates were incubated for 24/48 h at 30 °C for *Pseudomonas* spp. and 37 °C for *Lactobacilli* spp., sulfite-reducing anaerobic bacteria, total Coliform, and *E. coli*. Sulfite-reducing anaerobic bacteria were incubated in anaerobic conditions using a gas generating kit (Oxoid-Thermofisher, Milan, Italy).

2.5. ^{1}H-NMR analysis

A trichloroacetic acid extraction (TCA) was performed on all fish samples, by modifying the procedure set up by Pena-Pereira et al. (2010). For this purpose, 0.5 g of fish muscle was blended with 3 mL of 7% (w/w) trichloroacetic acid solution (TCA) by Ultra-Turrax (14,000 rpm, 20 s). An aliquot of homogenate (1 mL) was centrifuged at 18,670 g for 10 min at 4 °C. The supernatant (700 μl) was added with 100 μl of 10 mM 3-(Trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (TSP). Then, the pH was adjusted to 7.00 ± 0.02 using 9 mol/L or 1 mol/L KOH solutions in an Eppendorf microfuge tube. After centrifugation (10 min, 18,670 g at 4 °C), 650 μL of supernatant were transferred to an NMR tube for analysis. To quantify the concentration of each molecule, 100 μL of maleic acid (9.26 mM) were added as an internal reference to the sample treated at 400 MPa stored for 6 days.

To register ^{1}H-NMR spectra an AVANCE III spectrometer (Bruker, Milan, Italy) was employed, operating at a frequency of 600.13 MHz and equipped with the software Topspin (ver. 3.5). ^{1}H-NMR spectra were acquired using the CPMG pulse sequence, with suppression of the solvent's signal and the following key parameters: the size of fid: 32 k,

number of scans: 16, number of dummy scans: 16, spectral width: 12 ppm, acquisition time: 2.28 s, delay d1: 5 s. Phase, baseline, and chemical shift of each NMR spectrum were adjusted in Topspin, by considering the methyl group of alanine for the latter task. For any further processing, the spectra were converted to ASCII format through the script convbin2asc.

2.6. NMR data processing

Each ^{1}H NMR spectrum in ASCII format was further processed utilizing scripts in R (version 3.6) language developed in-house. For quantification, the spectrum from the sample treated at 400 MPa and stored for 6 days was considered as a reference, because characterized by a median solutes/water ratio, as assessed by probabilistic quotient normalization (PQN) (Dieterle et al., 2006). Any other sample was normalized towards the reference by applying PQN once more. The concentration of each molecule in each spectrum was calculated from the area of one of its signals, measured by global spectra deconvolution, implemented in MestReNova software (Mestrelab research S.L. Santiago De Compostela (Spain) - version 14.2.0–26256), This was done after applying a line broadening of 0.3 and a baseline adjustment by Whittaker Smoother procedure. Further details are given in our previous article (Lan et al., 2022).

2.7. Statistical analysis

Metabolites were considered as differently expressed due to treatment and storage time based on fold-change (|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 data analyses hierarchical clustering analysis (HCA) and robust principal component analysis (rPCA). The former was carried out by Origin Pro 2023 (OriginLab Corporation, USA), while the latter was carried out in R, by relying on the function PcaHubert (Todorov & Filzmoser, 2009).

The rate of nucleotides' overall degradation from IMP to hypoxanthine was evaluated by the H-value metric (LUONG et al., 1992), calculated as the ratio of hypoxanthine amount to the total amount of IMP, inosine, and hypoxanthine.

The statistical significance of variations in microorganism counts among the groups was assessed using SPSS software (version 8.0) through analysis of variance (ANOVA), followed by Tukey's post-hoc test ($p < 0.05$). In the R computational language, Spearman's correlation test was employed to investigate associations between microorganisms and metabolites, with corrections for multiple comparisons made following the method of Benjamini & Hochberg.

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. Microorganism analysis

The effects of the combination of storage and HHP treatments on the microbial profile of packaged grey mullets are described in Fig. 1. In all samples, *Salmonella* spp. and *Listeria monocytogenes*, were never detected. The microbiological criterion for determining the shelf life of the product was established at the point when the total mesophilic bacteria (TMB) reached a count of 6 log10 CFU/g, according to Regulation 2073/2005.

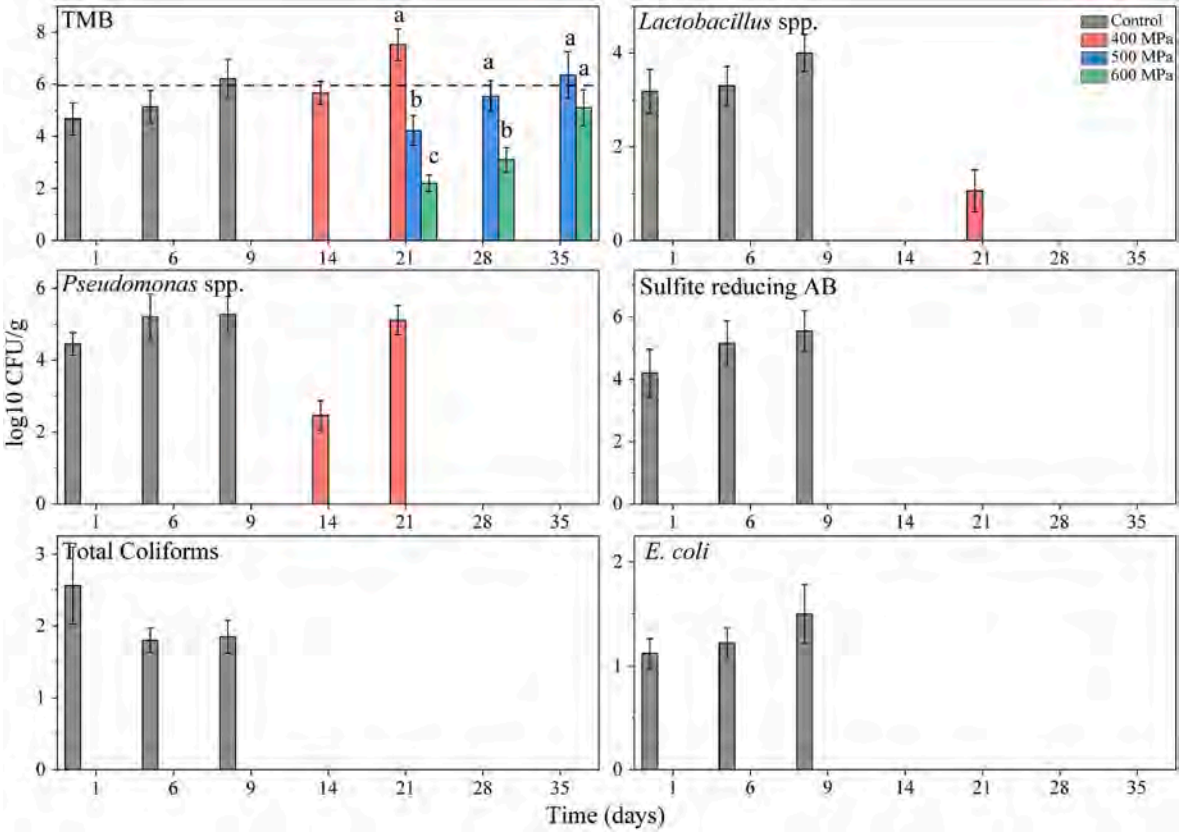

Fig. 1. Changes in microbial cell loads (log10 CFU/g) of total mesophilic bacteria (TMB), *Lactobacillus* spp., *Pseudomonas* spp., total Coliforms, sulfite-reducing anaerobic bacteria (AB), *E. coli* during storage of grey mullet products with different treatments. Different lowercase letters on the bar denote significant differences between means at $p < 0.05$.

The initial load of TMB in untreated samples appeared to be 4.67 log10 CFU/g, while no load could be registered in HHP treated samples. Following storage, TMB levels increased across all groups, albeit with a significantly reduced rate of increase ($p < 0.05$) observed with increasing pressure. The TMB in grey mullet samples treated at 400, 500, and 600 MPa reached the acceptability threshold of 6.0 log10 CFU/g on day 21, 35, and 35, respectively, compared to the 9 days of the control. In parallel, the cell load of *E. coli* was always under the detection limit.

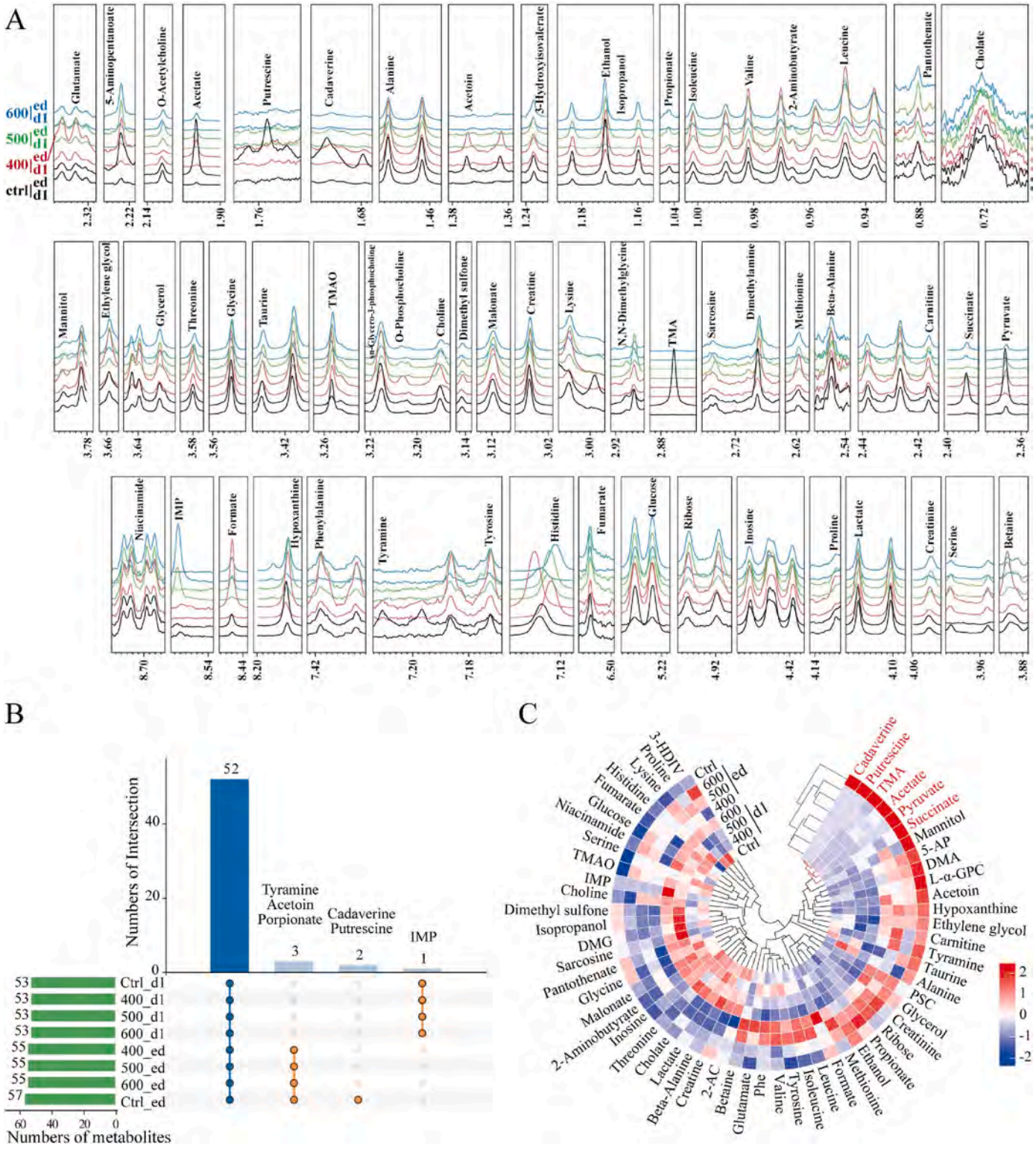

Fig. 2. (A) Examples of ^{1}H-NMR spectra registered on the control and HHP-treated grey mullet samples at day 1 (d1) and end day (ed). (B) UpSet plot of metabolites in the control group and HHP-treated groups at day 1 and end day of storage. (C) Heatmap summarizing the differences in the metabolites' profiles among the control group and to the HHP-treated groups, collected on day 1 and at the end day of storage. The end days for control, 400, 500, and 600 MPa were day 9, 21, 35, and 35, respectively. The red and blue colors indicate a high and a low expression of metabolites, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Interestingly, *Pseudomonas* spp. and *Lactobacilli* spp. were able to recover the insult due to the treatment with 400 MPa, reaching loads of 2.46 after 12 days and 1.06 after 19 days, respectively. In this respect, the samples treated with 400 MPa differed from those treated with 500 and 600 MPa, where these microbial groups went undetected.

3.2. Metabolome of grey mullet (Mugil cephalus) observable by ^{1}H-NMR along storage after HHP treatment

^{1}H-NMR was applied to evaluate the changes along storage in the grey mullet's metabolome in connection to the application of high

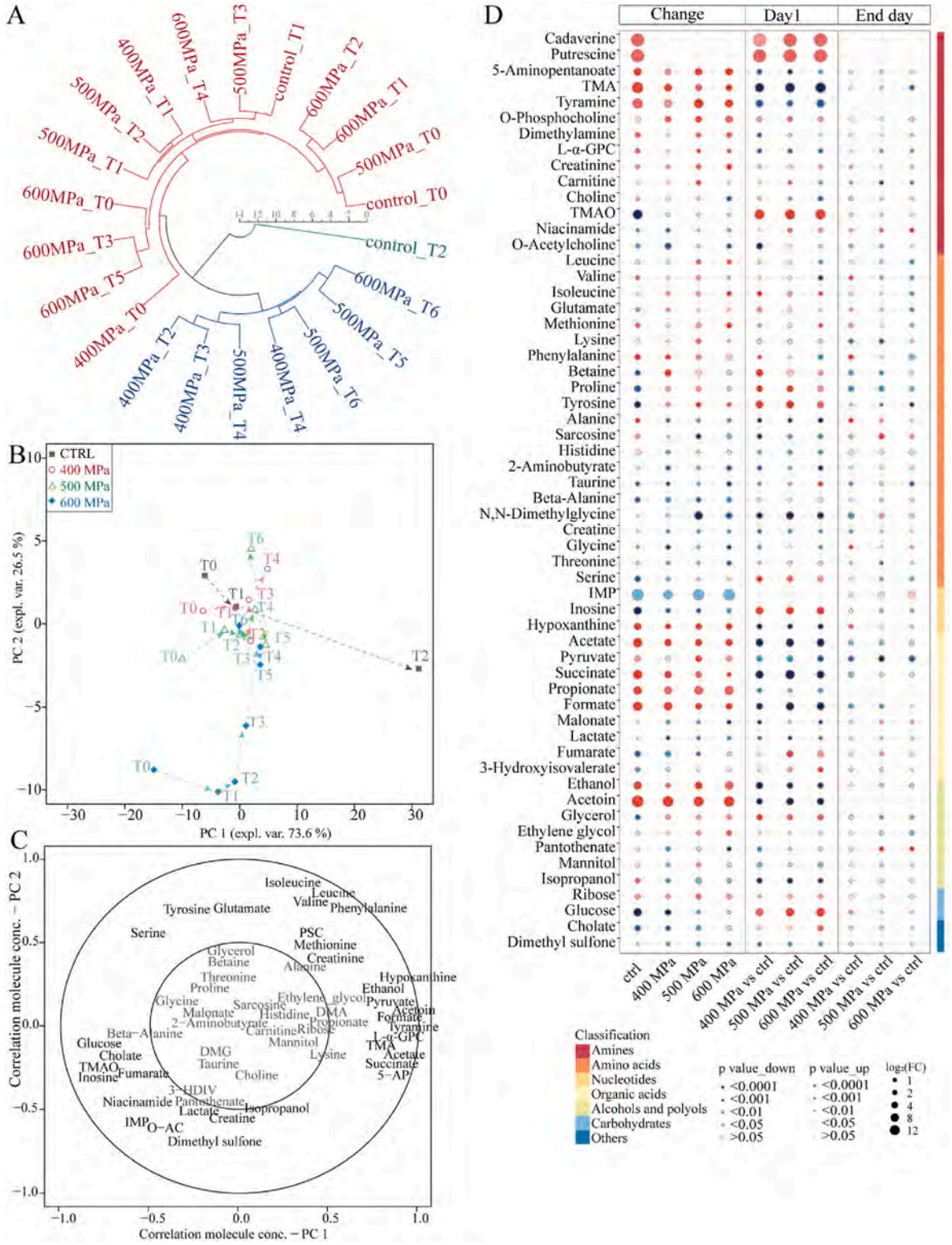

Fig. 3. (A) Hierarchical cluster analysis, (B) rPCA scores plot, and (C) rPCA loading plot of control group and HHP (400, 500, and 600 MPa) treated groups during chilled storage (T0, day1; T1, day6; T2, day9; T3, day14; T4, day21; T5, day28; T6, day35). (D) Bubble plot of *p* and fold change (FC). Change: end day vs day 1 in the same treated group. Day 1: day 1 HHP treated group vs day 1 control group. End day: end day HHP treated group vs end day control group.

hydrostatic pressures. Fig. 2A showcases spectra representative of all those registered for the current analysis. Fifty-eight metabolites were overall identified and quantified, including amino acids, amines, carbohydrates, nucleotides, and organic acids. The UpSet plot (Fig. 2B) generated by considering for each treatment the first and the last day of storage illuminates the molecules whose presence was linked to storage time. IMP could be detected in all groups only on day 1. On the opposite, three metabolites, namely tyramine, acetoin, and propionate, were detected in all groups only on the final day of storage, while two more metabolites, namely cadaverine, and putrescine, could be detected only on the final day of storage and only in untreated samples. A bird's eye representation of the overall metabolites' profiles granted by a heatmap (Fig. 2C) allows us to visually appreciate that on day 1 the metabolites' profiles of the treated samples were similar to those of untreated samples, while at the end day of storage the metabolites' overall profile of untreated samples appeared substantially different from the treated ones.

3.3. Multivariate analyses of grey mullet along storage after HHP treatment

To describe at a molecule-by-molecule level the chemical differences evidenced by the heatmap, an unsupervised hierarchical cluster analysis (HCA) was combined with a robust Principal Component Analysis (rPCA). The dendrogram from HCA (Fig. 3A) allowed us to visually appreciate that the chill-stored samples of grey mullet could be broadly classified into three groups, based on maximum Euclidean distance. The first cluster included only the untreated samples collected on the final day of storage. The second cluster was made by the treated samples collected towards the end of storage, namely those treated with 400 MPa and collected at days 9, 14 and 21, those treated with 500 MPa and collected at days 21, 28, and 35 and those treated with 600 MPa and collected at day35. The third cluster grouped the remaining treated samples, collected at the beginning of the storage. A closer look at the molecules driving these trends could be obtained through the generation of an rPCA model, that could be calculated by excluding cadaverine and putrescine, whose presence could be detected only in one type of sample. The rPCA's scoreplot based on the first two PCs is shown in Fig. 3B. For better clarity, the distribution of the samples along them is separately reported in Fig. S1. The main driver of the distribution of samples along PC1, accounting for 42% of the overall samples' variance represented by the model, appeared to be storage time. In detail, samples collected right after treatment appeared at low PC1 scores, while samples collected along storage appeared at progressively higher PC1 scores. In this respect, an extreme case was represented by untreated samples collected on the final day of storage, with the highest PC1 scores. PC1 scores seemed to be influenced partially also by treatment intensity. This can be inferred by noticing that the samples collected at T0 after being treated with 600, 500, 400, and 0 MPa appear in this very order along PC1 and that the same applies to the samples at T1 and at T2 too. Anyway, PC2 seemed to catch more clearly trends exclusively related to HHP treatment, with a particular emphasis on the treatment with 600 MPa, giving samples that systematically appear at the lowest PC2 scores, independently from the storage time.

Fig. 3C and S1C allowed us to appreciate that the molecules that mainly drove the trends along PC1 and increase with storage time are hypoxanthine, tyramine, acetoin, sn-glycero-3-phosphocholine, acetate, formate, TMA, succinate, pyruvate, ethanol, phenylalanine, 5-aminopentanoate and dimethylamine. On the contrary, those that decrease with storage were TMAO, inosine, glucose, cholate, fumarate, niacinamide beta-alanine, IMP, and serine. The molecules that drove the trends along PC2 and show high concentrations directly related to HHP treatments are dimethyl sulfone, O-acetylcholine, creatine, IMP, and isopropanol, while those inversely related to HHP are isoleucine, leucine, valine, glutamate, phenylalanine, tyrosine, and serine.

The high number of metabolites contributing to both trends and the partial overlap between trends prompted us to further evaluate the metabolome's changes via pairwise comparisons. Fig. 3D represents the three paired comparisons most suitable to obtain an overview of the trends linked to storage and HHP treatment. The first of them is the comparison, for each treatment, between concentrations on day 1 and on the final day of storage; the second and third of them are the comparisons between untreated and treated samples, respectively at day 1 and the end day of storage. In this context, the metabolites showing a $p < 0.05$ and a fold change higher than 2 could be considered the specific differential metabolites that mostly contributed to the intergroup separation. So selected, it is interesting to notice that all pairwise comparisons between control and treated samples showed several significant differences. Furthermore, compared with the control group, the HHP treated samples subjected to different pressures exhibited consistent trends in terms of changes in metabolite content. The molecules that drove the trends highlighted by the PC1 of the rPCA, storage-time related, can be systematically retrieved in the first column, while some of those highlighted by the PC2, treatment-related, can be retrieved among the molecules differentiating control and treated samples at T1, with particular emphasis on tyrosine, pyruvate, serine, and isopropanol.

3.4. Variations connected to different treatments in the intensity of metabolites involved in basal metabolism

Even if the portfolio of untargeted analyses described so far was found suitable to summarize trends connected to storage as influenced by HHP treatment, several metabolic pathways are known to directly cause quality deterioration along storage (Lan et al., 2022). It seemed appropriate therefore to set up observations targeted towards those metabolic pathways, namely nucleotide metabolism, TMA and tyramine production, and energy-related metabolism (Fig. 4).

The evolution of the nucleotides' concentration during storage for each treatment is illustrated in Fig. 4A. The IMP content on day 1 in the 500 MPa and 600 MPa treated groups was 2-fold and 7-fold that of untreated samples, respectively. This molecule appeared to degrade rapidly with storage so that it reached concentrations close to the limit of quantification in most of the groups after 9 days. The main differences between control and treated samples could be observed in inosine and hypoxanthine. In control samples, inosine concentration, the main nucleotide in all treatments on day 1, decreased sharply with storage, while its degradation product hypoxanthine showed a four-fold increase after 9 days. The same trends could be noticed for treated samples, but at a much slower pace and to a much lower extent. The rate of nucleotides' overall degradation from IMP to hypoxanthine could be effectively summarized by the H-value, as reported in the last graph of Fig. 3A. In control samples, H-value passed from 24.31% on day 1 to an unacceptable 93.44% on day 9. In sight of a practical use of H-value to estimate the acceptability of a product based on grey mullet, each HHP treatment on the final day of storage led to an H-value not higher than 55%.

Focusing on the biogenic amines (Fig. 4B and C), a consistent increase was observed with storage in both TMA and tyramine concentrations across all groups, much higher in the control than in the HHP treated group. As a confirmation, similar but opposite trends were observed in the concentrations of their precursors TMAO and tyramine. This was very clear in the control group, but more blurred in treated samples.

In the context of energy-related metabolism, the concentration of glucose exhibited a generalized decrease with storage, while its catabolites pyruvate, lactate, and ethanol increased. Notably, the rate of change for these metabolites was significantly (FCs >2, $p < 0.05$) reduced following any HHP treatment, in comparison to the control group.

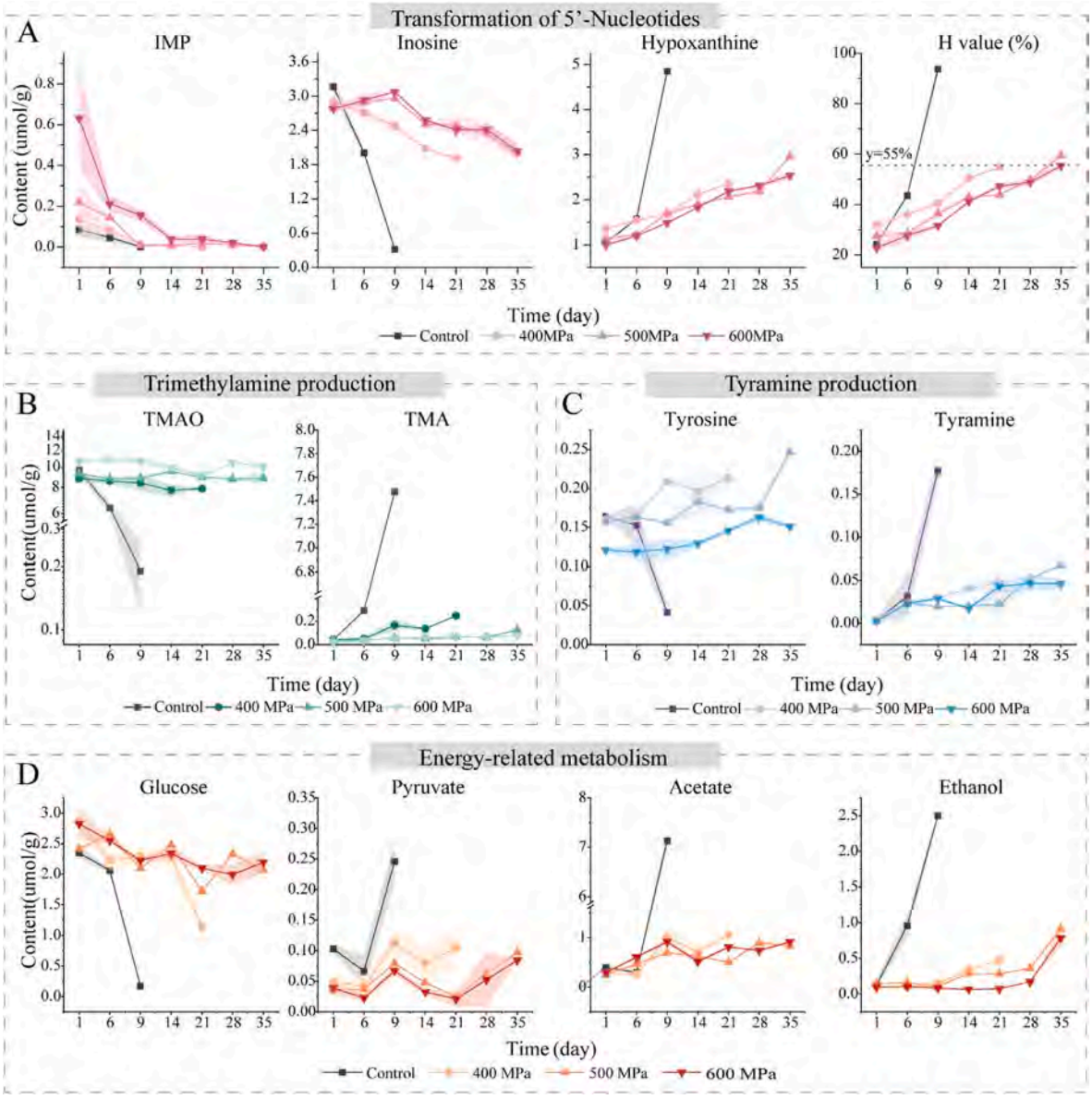

Fig. 4. Variations in the content of (A) 5′-Nucleotides transformation, (B) trimethylamine production, (C) tyramine production, and (D) energy-related molecules.

3.5. Pathway analysis of differential metabolites

To highlight the metabolic pathways mainly responsible for the metabolites' changes observed along storage in connection to HHP treatments, a two-step approach was designed. Firstly, a volcano plot was generated to evidence *p*-value and fold-change of the differences between the first and last days of storage for control group (Fig. 5A). These differences were compared to those due to the HHP treatments in further 3 volcano plots (Fig. 5B–D). Metabolites were defined as differentially expressed with a $p < 0.05$ and FCs >2.0 or <0.5. The observation of the same trends from two perspectives makes many of the distinctive metabolites in the first comparison appear also in the subsequent comparisons. For example, TMA, acetate, tyramine, ethanol, succinate, pyruvate, hypoxanthine, cadaverine, and putrescine showed an upregulation with storage in the control group, so that the same molecules appeared downregulated by the HHP treatments in comparison to the control samples.

The molecules overall highlighted by the volcano plots served as a basis for pathway analyses, performed on the control and on the HHP treated samples (Fig. 5E–F), by considering a $p < 0.05$ and a path impact >0 as limits for significance. As shown in Fig. 5E, there were 11 pathways significantly altered by the storage of untreated grey mullet. The disturbed pathways were tyrosine metabolism, pyruvate metabolism, glycolysis/gluconeogenesis, butanoate metabolism, citrate cycle (TCA cycle), phenylalanine metabolism, sulfur metabolism, methane metabolism, Alanine, aspartate and glutamate metabolism, glyoxylate and dicarboxylate metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, and lysine metabolism. The HHP treatments appeared to significantly alter the same pathways during the storage, except lysine metabolism.

3.6. Correlations between metabolites and microorganisms

To reveal the mechanisms underlying the preservation effects of HHP on fish muscle during chilled storage, we calculated the correlations among the screened metabolites in untreated and treated samples (Fig. 6A and B). As shown in Fig. 6B, in untreated samples 5-aminopentanoate, cadaverine, putrescine, TMA, acetate, succinate, hypoxanthine, ethanol, tyramine, and acetoin were significantly negatively correlated with tyrosine, serine, glucose, TMAO, cholate and pyruvate. The same

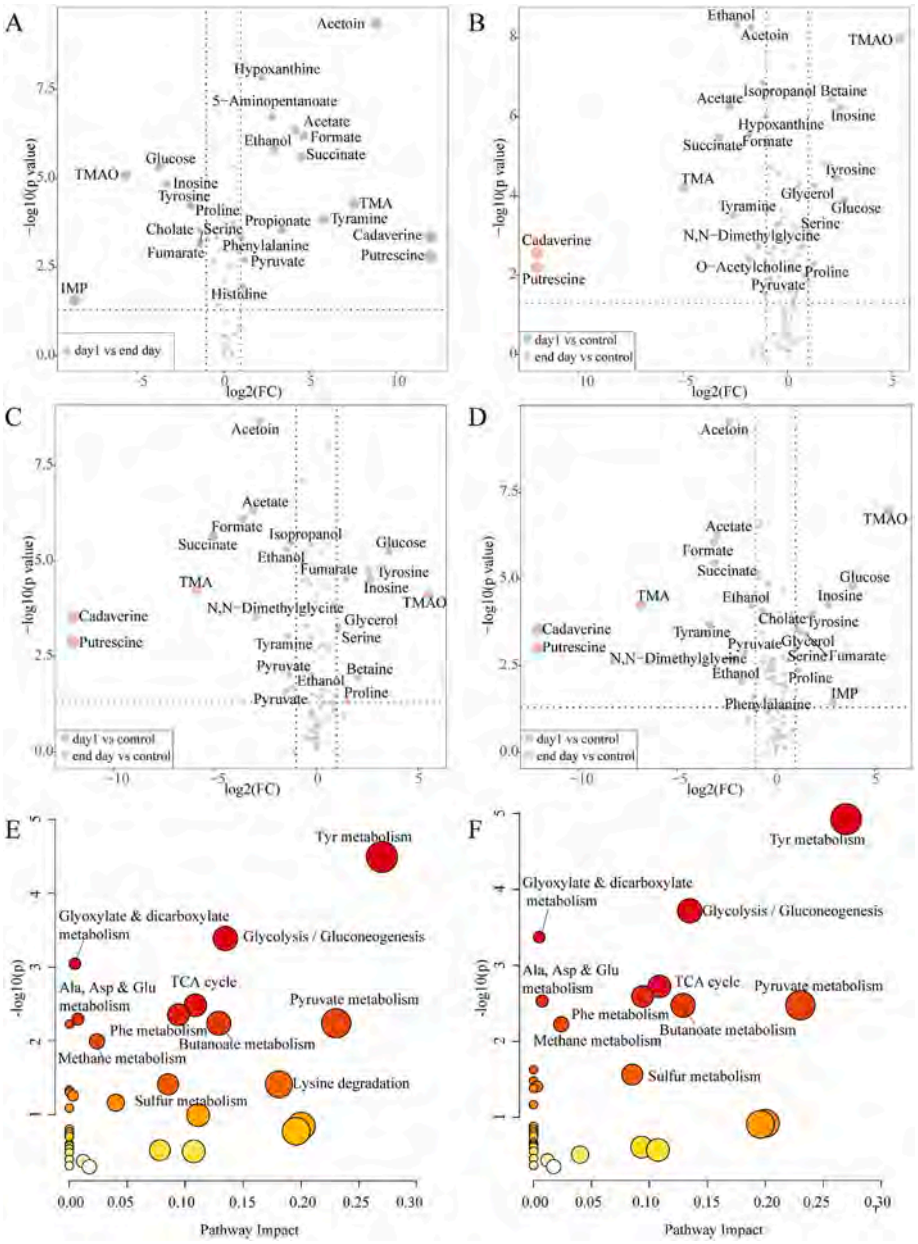

Fig. 5. Volcano plots (A–D) to compare the metabolites' profiles of grey mullet between the first and the last days of storage. The dotted lines evidence the value of 0.05 for p and the values of 2.0 and 0.5 for FC. The size indicate |log2 (FC)|. (A) control group (B) 400 MPa group (C) 500 MPa group (D) 600 MPa group. Corresponding pathway analyses focusing on control (E) and treated groups considered together (F).

molecules did not show significant correlations in the HHP treated samples. To gain deep knowledge of the relationship between differential metabolites and microorganisms, the results of Spearman's correlations test between total mesophilic bacteria (TMB) cell loads (log10 CFU/g) and metabolites are shown in Fig. 6C. TMAO, inosine, fumarate, and glucose were significantly negatively correlated with all considered microorganisms. We found positive correlations, in parallel, for TMA, hypoxanthine, succinate, and pyruvate. We also observed a significant positive correlation of energy-related metabolites and biogenic amines with all the considered microorganisms.

3.7. A potential metabolic pathway map of the cold-stored grey mullet products with HHP

The metabolic changes in grey mullet treated with HHP during chilled storage are summarized in Fig. 7, based on KEGG metabolic pathway analysis and previous studies (Chen et al., 2023; Wang et al., 2019). Five metabolic pathways were proposed, namely amino acid metabolism, carbohydrate metabolism, pyruvate metabolism, nitrogen metabolism, and nucleotide metabolism.

Both increasing and decreasing trends in concentrations of amino acid were observed in the chill-stored grey mullet with different treatments from the first day of storage to the last. For instance, the levels of some amino acids (betaine, proline, phenylalanine, and tyrosine) increased on the last day in HHP treated groups and decreased in control group. The opposite result was observed in the levels of N,N-Dimethylglycine, and histidine. Biogenic amines can work as biochemical indicators of fish quality and freshness. The typical spoilage-inducing compounds TMA, tyramine, putrescine, and cadaverine were detected in our study. The highest levels of above biogenic amines were reached by the untreated samples on the last day of storage.

Glucose is the major carbohydrate in grey mullets, which serves to

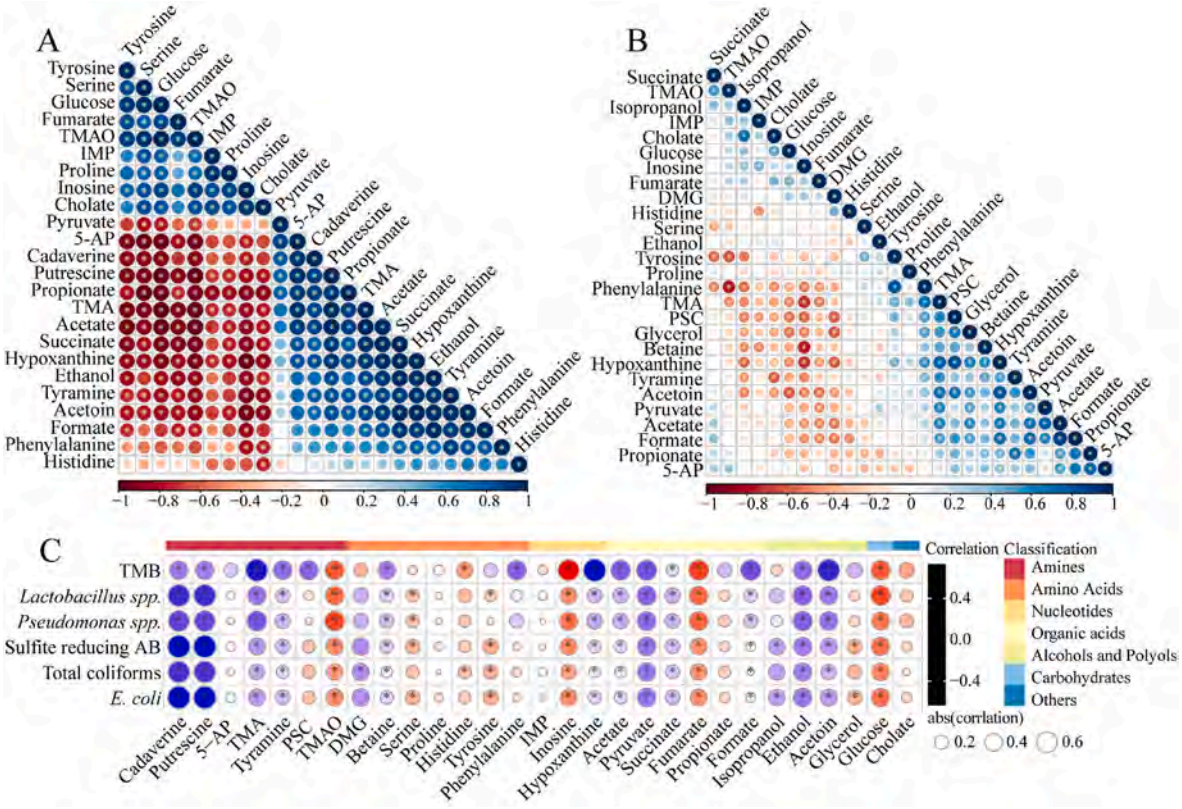

Fig. 6. Heatmaps of the correlation between the differential metabolites in control group (A) and treated group (B) and the correlation between the microorganisms and differential metabolites in all groups (C). * represent $p < 0.05$.

spoilage bacteria as a carbon source entering the TCA cycle and pyruvate metabolism to generate energy. As products of pyruvate metabolism and the TCA cycle, organic acids like succinate, acetate, and formate accumulate along storage, especially in untreated samples.

Nitrogen metabolism might lead to the deterioration of fish freshness, through the production of biogenic amines such as TMA. As shown in Fig. 6, TMA generally exhibits an increased trend during chill storage, while HHP treated samples have significantly lower TMA levels at the end day of storage, compared to the control group. It seems, therefore, that fish freshness could be maintained by HHP treated samples through the interruption of the nitrogen metabolism.

Molecules related to nucleotides metabolism were also found in grey mullet products during chilled storage. This is the process of the autolytic decomposition of ATP and the formation of its products, which include ADP, AMP, IMP, inosine, and hypoxanthine. In our study, 600 MPa treated samples have the highest levels of IMP on day1. Meanwhile, HHP treatment slowed down the formation of hypoxanthine from inosine.

4. Discussion

Grey mullets are highly esteemed in all the regions where they are fished, both fresh or processed as salted or smoked fillets (Khemis et al., 2019). Typically, ice storage is the only preservation method during processing, transport and marketing (Ninan & Zynudheen, 2014). Given its limited effectiveness in extending shelf life, these items are considered perishable, deteriorating rapidly due to the metabolic activity of specific spoilage organisms. Hence, it is imperative to develop strategies aimed at extending their shelf life. In this study, we utilized HHP to extend the shelf life of grey mullet products, as it has been demonstrated as an effective non-thermal method for maintaining the quality and freshness of diverse seafood products by controlling the development of various microbial groups (Zhao et al., 2019). The effects of HHP treatments on preservation have been investigated across various seafood products, revealing a high variability in outcomes due to differences in process parameters and the specific seafood species (Ekonomou & Boziaris, 2021). The application of HHP extended the microbial shelf life of grey mullet products from 9 days up to as much as 35 days. Compared to untreated samples, the reduced TMB levels in HHP treated samples indicated that HHP had a significant inactivating ($p < 0.05$) effect on spoilage microorganisms of grey mullets. The marked inactivating activity was evident also in the trends of single species.

The present metabolomic study by ^{1}H-NMR allowed us to monitor the levels of all the molecules needed to evaluate the nucleotides' degradation freshness index known as H-value. This index, based on the ratio between IMP and its degradation products, is one of the primary measures established to represent fish freshness with a single value. In the present study, the H-value was the most accurate indicator of the freshness of grey mullet, due to the rapid degradation of IMP to inosine. Similar results were obtained by Karim et al. (2019) for haddock (*Melanogrammus aeglefinus*) and herring (*Clupea harengus*). As shown in Fig. 3, the H-value in treated samples increased slowly but steadily over time. The trends of the single nucleotides allowed us to appreciate that the main drivers of its increase were inosine and hypoxanthine concentrations. HHP appeared therefore to slow the rate of nucleotide degradation, with a specific reference to inosine. This effect might be due to the reduced activity of nucleoside phosphorylase enzyme, responsible for the degradation of inosine to hypoxanthine (Karim et al., 2019). For practical applications of H-value, it is noteworthy that when the microbial load reached the acceptability threshold, the H-value was around 55% in all the samples analyzed. Thus, 55% could be suggested as the limit for fish acceptability.

The loss freshness of seafood products, as perceived by consumers in terms of fishy off-odor, is strongly associated with the presence of TMA, formed by the breakdown of TMAO mainly due to bacterial activity (Dalgaard, 1995). In this study, TMA of untreated samples increased by

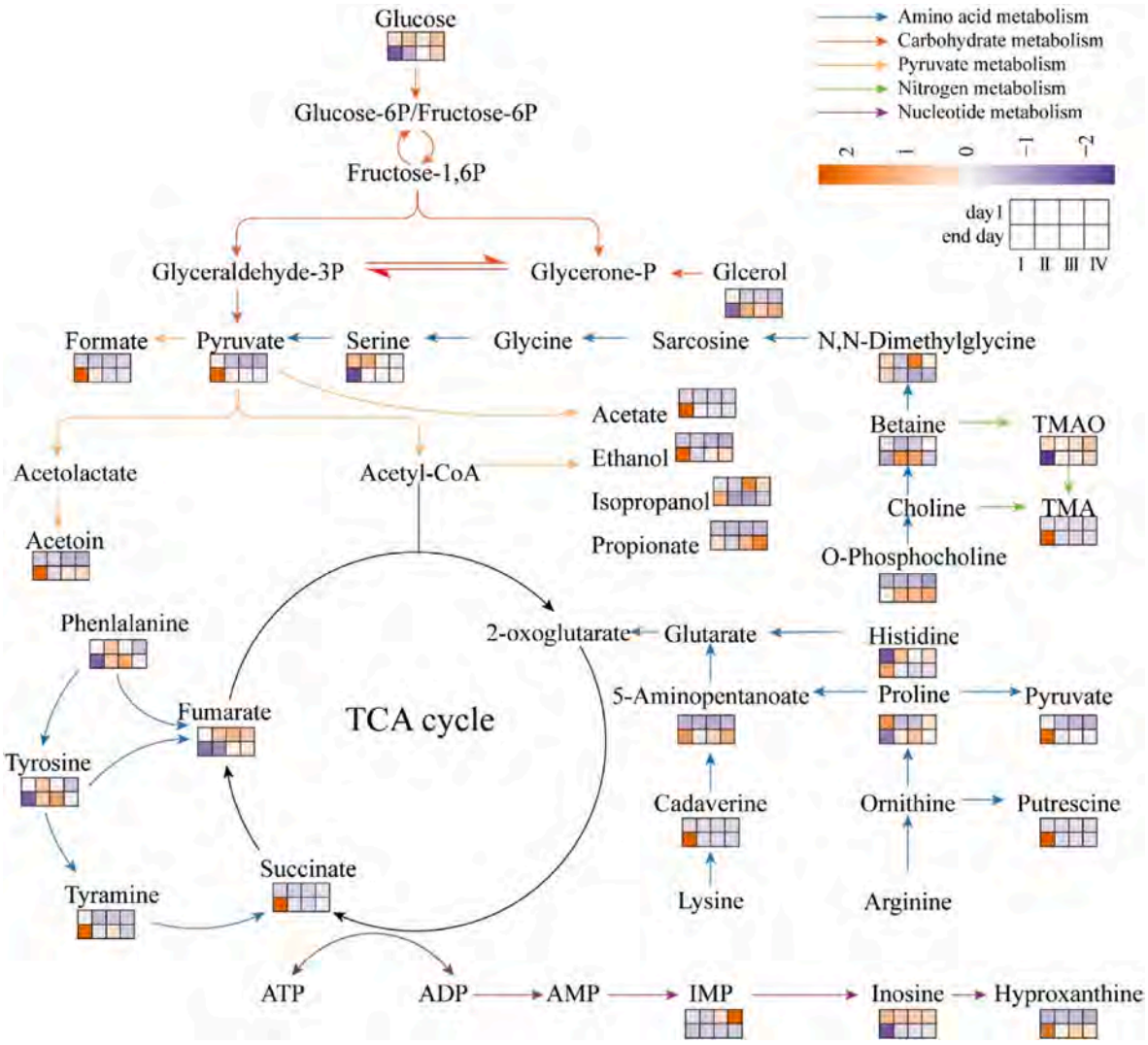

Fig. 7. Proposed schematic of metabolic alterations of differential metabolites in grey mullet under different treatments during chill storage. Note: I, control; II, III, and IV represent chill-storage grey mullet with 400, 500, and 600 MPa HHP, respectively. Colors from purple to orange indicate the gradual increase in metabolite concentrations extracted from different treatments (I-IV) on the first and last day.

4.7 mg/100g per day from day 1 to the end of storage. In marked contrast, in HHP-treated samples the increase rate was lower than 0.1 mg/100g per day, suggesting that HHP could effectively delay the development of off-odors related to TMA buildup. Similar to our findings, Reyes et al. (2015) reported that TMA content in 450 and 550 MPa treated for 3 or 4 min Chilean jack mackerel increased slowly and remained below 4 mg/100g. Integrating the results of pathway analysis, the reduced rate of TMA accumulation may be attributed to HHP's interference with microbial nitrogen metabolism, thereby diminishing the decline of the perceived fish freshness (Bennour et al., 1991; Erkan & Özden, 2008).

A hallmark of fish spoilage is the accumulation of biogenic, resulting from proteolysis and the activity of microbial amino acid decarboxylases during storage. This study has shown that ^{1}H-NMR is able to quantify simultaneously the three main biogenic amines putrescine, tyramine, and cadaverine. All the tested HHP treatments significantly reduced their accumulation so much that two of them, putrescine and cadaverine, remained below the detection limit during the entire storage time. This finding is consistent with the study by Kateřina Matějková et al. (2013), which observed that HHP treatments (300 and 500 MPa, 10 min) slowed the accumulation of putrescine, tyramine, and cadaverine in vacuum-packed trout flesh. Putrescine and cadaverine are produced by serval spoilage bacteria, including *Shewanella* spp., *Lactobacillus* spp., and *Pseudomonas* spp. (Ghaly et al., 2010; Visciano et al., 2020). The absence of detectable levels of *Lactobacillus* spp. and *Pseudomonas* spp. in several treated samples suggests that the reduced accumulation of biogenic amines was a consequence of the deactivation of these specific bacteria. Additionally, HHP may inhibit decarboxylase activity, which is responsible for the formation of biogenic amines (Ganjeh et al., 2024).

During storage, microbial metabolism of carbon source compounds plays an important role in the spoilage of fish and crustaceans. Glucose, the major carbohydrate in the grey mullet, is converted to pyruvate through glycolysis. In turn, pyruvate is converted to organic acids like acetate, formate, and succinate as well as to alcohols such as ethanol, through pyruvate metabolism and TCA cycle. In the present study, the higher levels of glucose and the lower levels of organic acid and alcohols in HHP treated groups may indicate the inhibition of glycolysis by HHP (Liu et al., 2023). Consistently, Jin Yue et al. (2016) observed a significant reduction in succinate and lactate levels in squid muscles following HHP treatment. These findings suggest that HHP treatments delayed the accumulation of organic acids and alcohols through pyruvate metabolism and TCA cycle, which are known to lead to unfavorable tastes and odors in fish products (Hara, 1994).

One positive aspect of ^{1}H-NMR is its ability to quantify molecules

beyond those targeted prompted by prior knowledge. The surprising outcome of these untargeted observations is that different levels of HHP led to similar metabolic profiles of grey mullet. This was evident in the volcano plot and key pathway analysis, which showed that the same metabolites were screened and the same key pathway were affected by different levels of HHP treatments. Therefore, different levels of HHP treatment seemed to affect the metabolism of chill-stored grey mullet product in similar ways. Off-odors and off-flavors related to spoilage of seafood could be a consequence of the amino acids', nitrogen's, and nucleotides' metabolisms (Liu et al., 2023; Lou et al., 2021). In this study, HHP treatments induced significant modifications in these pathways throughout the storage of untreated grey mullet products, with the exception of lysine metabolism. As a result, HHP treatments reduced the correlation coefficients among key metabolites, especially those associated with freshness such as TMA, inosine, and hypoxanthine, to values around 0.6.

Previous research has found that microbial activity induces metabolic changes in seafood (Lan et al., 2022; Lou et al., 2023). Hence, it is intriguing to clarify the relationship between the considered microorganisms and differential metabolites. These correlations appear to group the differential metabolites into three categories: those significantly correlated with all microorganisms, those correlated with some or one species of microorganisms, and those not significantly correlated with any microorganisms considered in this study. As many metabolic pathways regulate the concentration of these metabolites, it is hard to give a single biological explanation. Anyway, metabolites such as TMA, TMAO, nucleotides, acetate, and succinate, associated with fish freshness, showed significant correlations with microorganisms, indicating that the deterioration of freshness is due to microbial activity. Furthermore, HHP treatments efficiently reduced the count of microorganisms and maintained the overall freshness of grey mullet.

5. Conclusions

This study is the first to investigate the role and underlying mechanisms of metabolite alterations in cold-stored grey mullet products treated with HHP. As demonstrated in this study, HHP exhibits several positive effects, including bacteriostatic properties that reduce bacterial counts and the ability to influence the formation of volatile compounds. These findings highlight HHP as an efficient approach for preserving the freshness of fish. Moreover, this study successfully revealed metabolic changes in the grey mullet products with HHP treatments. Overall, HHP significantly ($p < 0.05$) slowed down the production of spoilage-related molecules such as TMA, tyramine, hypoxanthine, and ethanol. Further metabolites' analyses suggested that HHP influenced these key pathways of untreated chill-stored grey mullet products, except lysine metabolism. This underscores the need for a preservation method capable of effectively inhibiting lysine metabolism, highlighting an avenue for further research and development. Furthermore, this study shows that NMR-based metabolomics strategy can effectively identify processing effects on food matrices and provide a theoretical framework for future studies of their mechanisms of action and adjustment of parameters. Although this study focuses on grey mullet products, future research should explore whether these findings apply to other fish species or food types. Moreover, there exists a necessity for further refinement of HHP parameters, encompassing pressure levels, treatment durations, and temperatures, to optimize its efficacy in preserving fish freshness while minimizing any negative effects on taste and nutritional quality.

CRediT authorship contribution statement

Qiuyu Lan: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Silvia Tappi:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Giacomo Braschi:** Writing – review & editing, Software, Methodology. **Gianfranco Picone:** Writing – review & editing, Methodology, Formal analysis. **Pietro Rocculi:** Writing – review & editing, Resources, Data curation, Conceptualization. **Luca Laghi:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2024.104539.

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