Food Control 144 (2023) 109341

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Metabolic and enzymatic changes of *Shewanella baltica* in golden pomfret broths during spoilage

Xiaowei Lou [a,b], Yaowen Hai [a,b], Yi Le [a], Xinli Ran [a,b], Hongshun Yang [a,b,*]

[a] Department of Food Science and Technology, National University of Singapore, Singapore, 117542, Republic of Singapore
[b] National University of Singapore (Suzhou) Research Institute, 377 Lin Quan Street, Suzhou Industrial Park, Suzhou, Jiangsu, 215123, PR China

ARTICLE INFO

Keywords:
Shewanella baltica
Fish
Spoilage
Specific spoilage organism
Bacterial enzyme
Metabolomics
NMR
Carboxypeptidase
Aminopeptidase
Endoprotease
Nucleotidase
Broth
Seafood
Metabolite
Omics
Microbial safety
Storage
Preservation
Pathway
Food safety

ABSTRACT

Shewanella baltica was identified as a specific spoilage organism in golden pomfret. Its spoilage potential and activity have been reported to be closely related to its enzymes. In this work, the main enzymes of *S. baltica* were reported, and enzyme activities as well as changes of intracellular metabolites of 3 strains (ABa4, ABe2, BBe1) in fish broth during spoilage were determined. Totally, 47 enzymes were identified, amongst which cysteine desulfurase and Lon protease were related to the spoilage potential of *S. baltica*. Aminopeptidases and endoproteases in *S. baltica* might make contribution to the spoilage than carboxypeptidases, especially during early spoilage (0–4 d), in which aminopeptidase activity increased by 31.44%–342.01%. In this period, amino acids, organic acids and sugars increased in strain ABa4 and ABe2 (up to 8.73 $\log_{1.5}$ fold change), due to that they were transferred into the cell matrix for bacterial proliferation and activity. In the later spoilage (4–10 d), proteases and nucleotidases were still produced but secreted out of the cell, accompanied by metabolite consumption. The enhanced levels of metabolites in strain BBe1 during later spoilage might be due to a stronger metabolite intake than the consumption. The altered metabolites were mainly involved in amino acid metabolisms, aminoacyl-tRNA biosynthesis and purine metabolism, which were connected with the spoilage activity of *S. baltica*. This study investigates the spoilage-related enzymes, metabolites and metabolisms of *S. baltica*, providing information for the spoilage controlling of seafood.

1. Introduction

Fish is regarded as an affordable and important protein source in daily diet, accounting for around 17% of the intake of animal protein globally (FAO, 2020). In Singapore, 16 kg of fish were consumed per capita in 2020, while only 8% of fish was produced in local farms (AVA, 2021). To enhance food security, a lot of offshore fish farms were built and golden pomfret (*Trachinotus blochii*) was spawned successfully in these farms (Feng et al., 2017).

On the other hand, fish is a highly perishable food after death and during chilled storage, in which microbial activity is proved as an essential cause (Parlapani et al., 2017; Prabhakar et al., 2020). *Shewanella baltica* was identified to be one of the specific spoilage organisms (SSOs) in spoiled fish, such as Atlantic horse mackerel (Alfaro et al., 2013) and large yellow croaker (Zhu et al., 2016). This bacterium has the capacity to degrade fish cytoskeleton, impair fish texture, and produce trimethylamine (TMA) as well as biogenic amines, resulting in "fishy" off-flavour (Zhu et al., 2015).

Several studies investigated that the high spoilage ability of *S. baltica* was associated with the enzymes inside the strain or secreted by the

Abbreviations: SSO, specific spoilage organism; TMA, trimethylamine; TMAO, trimethylamine-*N*-oxide; TVB-N, total volatile basic nitrogen; ATP, adenosine triphosphate; HxR, inosine; Hx, hypoxanthine; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; TVC, total viable count; NMR, nuclear magnetic resonance; LB, Luria-Bertani; PBS, phosphate-buffered saline; HEPES, *N*-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); TMT, tandem mass tag; LC-MS/MS, liquid chromatography with tandem mass spectrometry; PEP, posterior error probability; DMSO, dimethyl sulfoxide; TSP, sodium 3-trimethylsilyl [2,2,3,3-d_4] propionate; PCA, principal component analysis; PC, principal component; TCA, tricarboxylic acid.

* Corresponding author. Department of Food Science and Technology, National University of Singapore, Singapore, 117542, Republic of Singapore.
E-mail address: fstynghs@nus.edu.sg (H. Yang).

https://doi.org/10.1016/j.foodcont.2022.109341
Received 6 June 2022; Received in revised form 6 August 2022; Accepted 25 August 2022
Available online 29 August 2022

strain. Lou et al. (2021) illustrated that the productions of amino acids, tyramine, acetate, succinate, inosine (HxR) and hypoxanthine (Hx) in the fish block could be due to the protease, decarboxylase and nucleotidase in *S. baltica*. Li et al. (2020) found a list of genes that encode spoilage-related enzymes in *S. baltica* 128, including 3′(2′), 5′-bisphosphate nucleotidase (*cys*Q), ornithine decarboxylase (*spe*C), trimethylamine-*N*-oxide reductase (*tor*C), general secretion pathway protein (*gsp*X), and serine protease production (*deg*S). Zhu et al. (2019) mentioned that *S. baltica* biofilm was able to secrete protease, trimethylamine-*N*-oxide (TMAO) reductase and lipases at low temperatures. Feng et al. (2021) observed an increase of extracellular protease activity of *S. baltica* in fish broths stored from 4 d to 6 d, accompanied by the enhanced levels of TMA, putrescine and total volatile basic nitrogen (TVB-N). The secreted proteases by spoilage bacteria can be grouped into endoproteases and exoproteases that are consisted of aminopeptidases and carboxypeptidases (Ahangari et al., 2021; Venugopal, 1990). Up to now, little information is available on the exactly enzymes in *S. baltica* and the changes of the activities of carboxypeptidases, aminopeptidases, endoproteases and nucleotidases in *S. baltica* during spoilage.

The spoilage activity of SSOs is defined as the quantitative ability to produce metabolites (Boziaris & Parlapani, 2017). These metabolites can be the precursors of off-flavour compounds or be consumed by SSOs for growth and respiration. In addition, the metabolic products are correlated with sensory properties and shelf life in fish products (Chouliara et al., 2004). During the chilled storage, SSOs metabolise free amino acids to biogenic amines, ammonia, sulfur compounds and organic acids, and induce adenosine triphosphate (ATP) degradation to form inosine and hypoxanthine (Gram & Dalgaard, 2002). Macé et al. (2013) stated that a mix of feet/cheese/sour/amines flavour was produced by *S. baltica* in salmon fillet. The metabolic changes in fish blocks and broths inoculated with *S. baltica* during storage were explored in our previous study (Lou et al., 2021), while the changes of intracellular metabolites of *S. baltica* during fish spoilage have not been evaluated.

Sterile fish broth is a useful fish model for bacterial spoilage study, since it contains fish nutrients and has been autoclaved to prevent the influence of original microflora on fish (Zhu et al., 2016). In this work, the main enzymes in three *S. baltica* strains were firstly identified and analysed using Gene Ontology (GO) functional analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analysis. Then strains were inoculated into sterile fish broths and stored for up to 10 d. Total viable count (TVC) and enzyme activities of *S. baltica* during spoilage were determined, and the changes of intracellular metabolites of *S. baltica* were tested using nuclear magnetic resonance (NMR). The objectives were to further understand the spoilage mechanism of *S. baltica* in fish, and to provide insight for spoilage controlling of seafood.

2. Materials and methods

2.1. Chemicals and reagents

Methanol-d4 was purchased from Cambridge Isotope Laboratories (Miami, FL, USA). Other chemicals (analytical grade) and kits were bought from Sigma-Aldrich (St. Louis, MO, USA). Luria-Bertani (LB) agar and LB broth were bought from Oxoid (UK).

2.2. Preparation of sterile fish broths

Golden pomfret was purchased and gutted in Song Fish Dealer Pte Ltd in Singapore. Fish were placed in an ice box and transported to the laboratory within 1 h after purchasing. Sterile fish broths were prepared according to our previous work (Lou et al., 2021) with slight modifications. Briefly, fish fillets were homogenised with distilled water in a ratio of 1:1. The mixture was filtered with double gauze and boiled for 5 min. After cooling and re-filtering, 0.10 M phosphate buffer was added to the broths, and the pH was adjusted to 6.50. Afterwards, the broths were autoclaved, cooled and centrifuged at 8000×*g* for 10 min at 4 °C. The supernatants were collected and used within 4 h.

2.3. Bacteria strains and culture condition

Three *S. baltica* strains (ABa4, ABe2, and BBe1) were previously separated from spoiled golden pomfret. The strains were firstly activated and transferred in LB broth at 4 °C and the discrete colonies were selected using LB agar. The colonies were re-cultured in LB broth and incubated at 4 °C to reach a concentration around 7 log CFU/mL for further analysis and inoculation.

2.4. Bacterial protein preparation and enzyme identification

Ten milliliters of LB broth with *S. baltica* enriched to 7 log CFU/mL were centrifuged at 8000×*g* for 10 min at 4 °C and the pellets were washed thrice with 10 mM phosphate-buffered saline (PBS, pH 7.2). Bacteria were lysed in the buffer (pH 8.0) containing 8 M urea and 50 mM *N*-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES). Samples were sent to Institute of Molecular and Cell Biology (A*STAR Research Entities, Singapore) for protein digestion, tandem mass tag (TMT) labelling, fractionation, liquid chromatography with tandem mass spectrometry (LC-MS/MS) and MS data analysis (Loo et al., 2020).

The main bacterial enzymes were selected with the following strategies: 1. The protein should be named as an enzyme; 2. The protein abundance should be top 500; 3. Posterior error probability (PEP) score should be higher than 80; 4. Mascot score should be higher than 1000.

2.5. GO and KEGG analysis

The GO functional analysis of selected enzymes was analysed using the STRING online software (https://string-db.org/), and then visualized with SIMCA-P+ (version 14.1, Umetrics, Sweden). KEGG pathways enrichment analysis was performed with the KOBAS online software (http://bioinfo.org/kobas/).

2.6. Inoculation and enumeration of S. baltica

Inocula were inoculated into sterile fish broths to reach a final level around 4 log CFU/mL. The inoculated broths were stored at 4 °C for up to 10 d.

Broth samples on days 0, 4 and 10 were serially diluted (1/10, v/v) with 0.1% peptone water. TVCs of *S. baltica* in fish broths were determined on LB agar with 6 d incubation at 4 °C, and expressed as log CFU/mL.

2.7. Determination of enzyme activities of S. baltica

2.7.1. Extraction of bacterial enzymes

Bacterial enzymes were extracted according to Wu et al. (2015) with slight modification. Inoculated broths on days 0, 4 and 10 were centrifuged at 8000×*g* for 10 min at 4 °C. Cell pellets were washed with 10 mM PBS (pH 7.2) for three times, resuspended in PBS to reach a level of 9 log CFU/mL, and lysed by sonication. After centrifugation for 10 min at 8000×*g*, the supernatants were collected as crude enzyme extracts.

2.7.2. Determination of carboxypeptidase activity

The assay of carboxypeptidase was determined based on the methods of Chen and He (2004) with slight modification. Hippuryl-L-phenylalanine (2 mM) was used as the substrate and dissolved in 0.05 M Tris-HCl with 0.5 M sodium chloride, pH 7.5. The substrate and the crude enzyme were pre-incubated at 25 °C. Afterwards, 200 μL of substrate and 10 μL of enzyme solution were mixed at 25 °C for 15 min and the absorbance at 254 nm was tested using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The enzyme mixed with

Tris-HCl buffer was set as the blank. One unit of activity was equivalent to the change of one absorbance at 254 nm.

2.7.3. Determination of aminopeptidase activity

Aminopeptidase activity was determined as described by Wu et al. (2015) with slight modification. The substrate medium (L-leucine-*p*-nitroanilide) was first dissolved in dimethyl sulfoxide (DMSO), then diluted to 2 mM with 10 mM PBS, pH 7.2. The enzyme extract (10 μL) and substrate (200 μL) were incubated at 37 °C in the microplate reader for 1h and the absorbance at 410 nm was tested. The blank was the mixture of enzyme extract and PBS. One unit of aminopeptidase activity corresponded to an absorbance change at 410 nm.

2.7.4. Determination of endoprotease activity

Endoprotease activity was examined according to Sinsuwan et al. (2008) and Kim et al. (2012) with slight modification. Reaction mixture contained 160 μL of 2 mg/ml azocasein in 0.1 M sodium phosphate buffer (pH 6.0), and 40 μL of the enzyme extract. After 1 h incubation at 40 °C in the microplate reader, the absorbance was read at 440 nm. The reaction mixture without azocasein was set as the blank. One unit of activity is equivalent to the change of one absorbance unit.

2.7.5. Determination of nucleotidase activity

Nucleotidase activity was determined by the orthophosphate liberation from nucleotides (Chróst, 1991; Sales & Santoro, 2008) with slight modification. The substrate (1 mM ATP in 10 mM PBS containing 3.8 mM $MgCl_2$, pH 7.2) and the crude enzyme were pre-incubated at 37 °C. Enzyme sample (50 μL) and ATP solution (50 μL) were mixed and incubated at 37 °C for 15 min and the liberated orthophosphate was assayed by a Phosphate Colorimetric Kit (MAK030, Sigma-Aldrich) according to the specification. The blank was the mixture without ATP. One unit of activity is equivalent to an absorbance change at 650 nm.

2.8. Preparation of metabolites, NMR analysis and spectral processing

Metabolite extraction in *S. baltica* was done according to Wang et al. (2022) with slight modification. Around 9 log CFU bacterial pellets were collected for each sample according to section 2.7.1. The collected pellets were mixed with 0.7 mL of ice-cold methanol-d4 containing 0.01% sodium 3-trimethylsilyl [2,2,3,3-d4] propionate (TSP, as internal standard), and treated with a frozen-thaw cycle for three times using liquid nitrogen to destroy the bacterial membrane. Samples were kept at −20 °C overnight. After the centrifugation at 12,000×*g* for 10 min at 4 °C, 600 μL of supernatant was transferred into a 5 mm NMR tube.

NMR spectra of extracts from *S. baltica* were performed at 25 °C on a Bruker DRX-500 NMR Spectrometer (Bruker Biospin, Rheinstetten, Germany) with a Triple Inverse Gradient probe at 500.23 MHz. The parameters of 1D 1H and 2D 1H–13C NMR spectra were identical to our previous work (Lou et al., 2021) and were analysed with TopSpin 4.0.7 software (Bruker Biospin, Rheinstetten, Germany).

The metabolites were identified with 1D and 2D spectra cooperatively according to databases of The Metabolomics Innovation Centre (http://www.hmdb.ca/), Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu/metabolomics) and related references. The regions of the water (4.69–5.00 ppm) and methanol (3.31–3.36 ppm) were excluded and NMR data were binned using Mestrenova (Mestreab Research SL, Santiago de Compostela, Spain) for integration. A heatmap was plotted using ClustVis web tool (https://biit.cs.ut.ee/clustvis/). Principal component analysis (PCA) plots and volcano plots were processed with SIMCA-P+ (version 14.1, Umetrics, Sweden). The involved pathways were analysed on MetaboAnalyst 5.0 online software (http://www.metaboanalyst.ca/).

2.9. Statistical analysis

All tests were performed in triplicate independently. Analysis of variance (ANOVA) was done by using Duncan's test in XLSTAT (AddinSoft, New York, USA). The difference of $P < 0.05$ was set as the significant difference.

3. Results and discussion

3.1. Main enzymes in S. baltica, GO and KEGG analysis

The summary of selected enzymes and exact names are shown in Fig. 1a and Table S1, respectively. Totally, 47 enzymes with high abundance and confidence in three *S. baltica* strains were identified, including 10 oxidoreductases, 12 transferases, 6 hydrolases, 3 lyases, 5 isomerases, 5 ligases, 4 translocases and 2 oxidoreductases/translocases. In our previous study, *S. baltica* showed a high spoilage ability to decompose nutrients in fish, especially proteins and nucleotides (Lou et al., 2021). To better understand the relationship between identified enzymes and the spoilage ability of our strains, GO functional analysis and KEGG pathways enrichment analysis were performed.

There are three categories of GO analysis for dominant enzymes in our *S. baltica*: biological process (Fig. 1b), molecular function and cellular component (Table S2). Compared with other categories, biological process showed a greater number of terms and more importance. The selected enzymes were distributed to 31 functions in the biological process category, with a large number of enzymes involved in the metabolic process (28 enzymes), cellular process (26 enzymes), cellular metabolic process (24 enzymes), nitrogen compound metabolic process (23 enzymes) and organic substance metabolic process (23 enzymes).

KEGG pathways enrichment analysis (Fig. 1c) indicated that the selected enzymes were primarily involved in metabolic pathways (16 enzymes), microbial metabolism in diverse environments (9 enzymes), biosynthesis of secondary metabolites (8 enzymes), carbon metabolism (8 enzymes) and biosynthesis of antibiotics (7 enzymes). Our enzymes were also correlated with several amino acid metabolisms, such as Ala, Asp and Glu metabolism, Trp metabolism, and Lys degradation (Fig. 1c).

The spoilage potential of *S. baltica* was related to metabolisms of trimethylamine, biogenic amines and sulfur-containing compounds (Fu et al., 2018). In our results, cysteine desulfurase was involved in the sulfur relay system, which might be responsible for H_2S production. In addition, proteins, amino acids and nucleotides can be converted to off-flavour compounds in seafood (Lou et al., 2021). The enzymes of amino acid and nucleotide metabolisms might also be connected with the spoilage potential and activity of *S. baltica*. Li et al. (2020) identified serine protease and nucleotidase in *S. baltica* 128 using complete genome sequencing. In our *S. baltica* strains, Lon protease, which was known as a kind of serine protease, might be the dominant protease for protein degradation during fish spoilage.

3.2. Changes of TVC and enzyme activities of S. baltica in fish broths during spoilage

Fig. S1 shows the TVC of *S. baltica* in fish broth on days 0, 4 and 10. It can be seen that the populations of three *S. baltica* strains on day 10 exceeded 7 log CFU/mL, which was regarded as the rejection limit for seafood according to the relationship between TVC and sensory scores (Mol et al., 2007). Three strains presented similar increasing growth trends during spoilage ($P < 0.05$), which was the same as our previous observation (Lou et al., 2021). This suggested that the fish broths inoculated with *S. baltica* could be used for simulating fish spoilage.

To further understand the effect of enzymes in *S. baltica* during spoilage, the activities of carboxypeptidases, aminopeptidases, endoproteases and nucleotidases on days 0, 4 and 10 were tested and shown in Fig. 2. During storage, carboxypeptidases showed low activities and no significant difference in three strains ($P > 0.05$). Aminopeptidase activity increased by 31.44%–342.01% from day 0 to day 4 ($P < 0.05$), but decreased from day 4 to day 10 ($P < 0.05$). Aminopeptidases in strain ABa4 had the highest activity after storage. The activity of

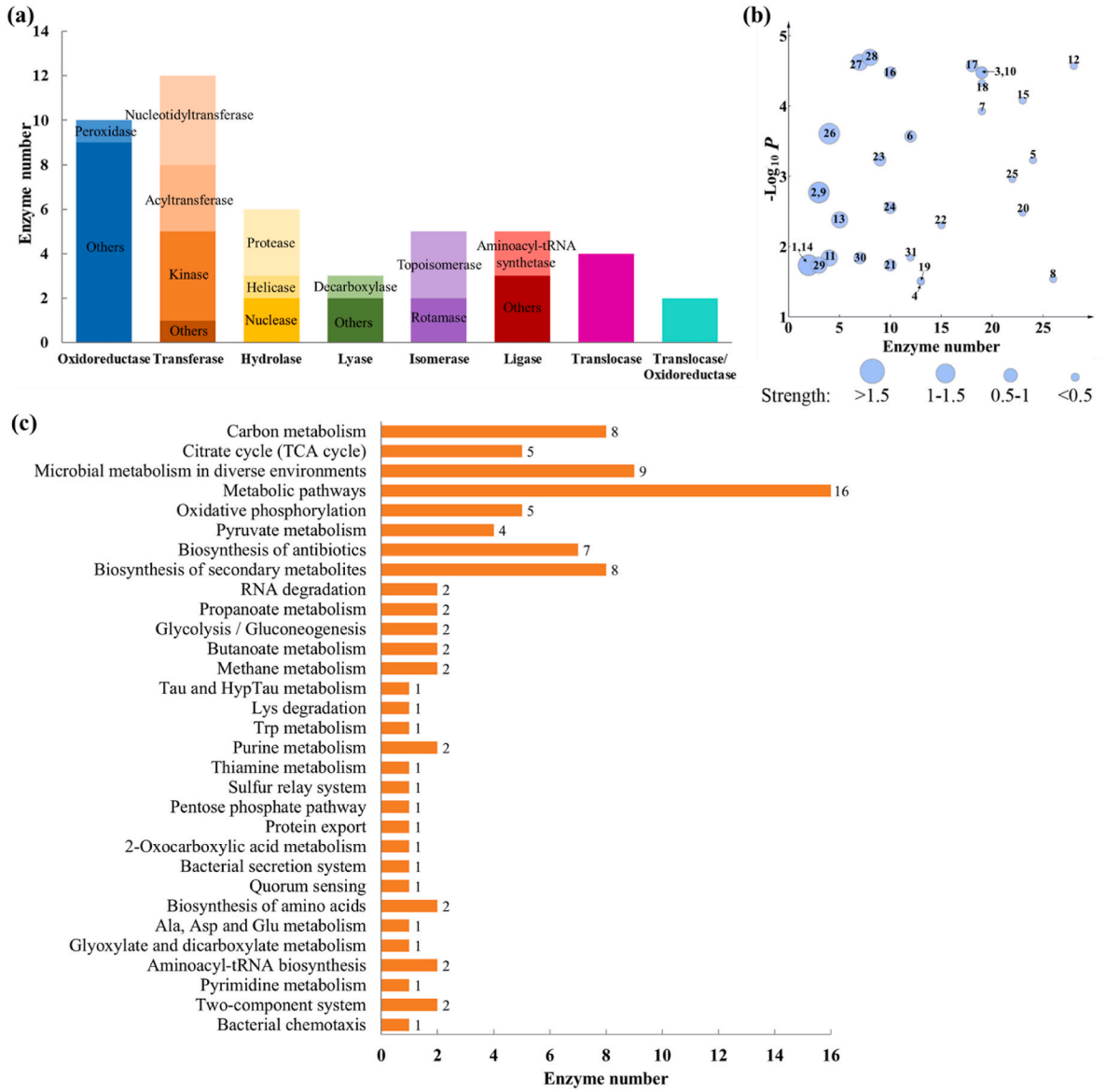

Fig. 1. Identified enzymes (a) in *S. baltica*, GO functional analysis associated with biological processes (b) and KEGG pathways enrichment analysis (c). Keys in (b): 1: acyl-CoA biosynthetic process; 2: ATP synthesis coupled proton transport; 3: Cellular aromatic compound metabolic process; 4: Cellular biosynthetic process; 5: Cellular metabolic process; 6: Cellular nitrogen compound biosynthetic process; 7: Cellular nitrogen compound metabolic process; 8: Cellular process; 9: DNA topological change; 10: Heterocycle metabolic process; 11: Macromolecule catabolic process; 12: Metabolic process; 13: Monovalent inorganic cation transport; 14: mRNA catabolic process; 15: Nitrogen compound metabolic process; 16: Nucleobase-containing compound biosynthetic process; 17: Nucleobase-containing compound metabolic process; 18: Organic cyclic compound metabolic process; 19: Organic substance biosynthetic process; 20: Organic substance metabolic process; 21: Organonitrogen compound biosynthetic process; 22: Organonitrogen compound metabolic process; 23: Organophosphate metabolic process; 24: Phosphate-containing compound metabolic process; 25: Primary metabolic process; 26: Purine ribonucleoside triphosphate biosynthetic process; 27: Purine ribonucleotide biosynthetic process; 28: Ribonucleotide biosynthetic process; 29: RNA catabolic process; 30: RNA metabolic process; 31: Small molecule metabolic process. Note: Strength = Log_{10} (the number of proteins observed in this experiment/the number of proteins expected in a random network of the same size).

endoproteases in strain ABa4 on day 0 showed a relatively high amount and significantly decreased during spoilage ($P < 0.05$), while it in strain BBe1 showed a contrary trend. The change of nucleotidase activity in strain ABa4 was similar to that of aminopeptidases, whereas the nucleotidases in strain BBe1 kept a relatively high activity during whole storage without significant change ($P > 0.05$). Strain ABe2 obtained a moderate level of endoprotease activity and lower activities of aminopeptidases and nucleotidases compared to strains ABa4 and BBe1.

Venugopal (1990) reported that exoproteases and endoproteases were representative hydrolytic enzymes secreted by spoilage microorganisms. Exoproteases break peptide bonds at the carboxy-terminal or at the amino-terminal, which are called carboxypeptidases and aminopeptidases, respectively. On the contrary, endoproteases cleave peptide bonds in the middle of the amino acid chain. Ge et al. (2017) detected high activities of protease in *S. baltica* after 24 h incubation. In our results, aminopeptidases and endoproteases might contribute more to the spoilage activity of *S. baltica*, especially during early spoilage stage. Strain ABa4 produced more aminopeptidases, while a higher amount of endoproteases was produced by strain BBe1 after 10 d chilled storage.

Nucleotidases are a group of hydrolytic enzymes that catalyses the hydrolysis of nucleotides into nucleosides and phosphates. Fish freshness can be evaluated by K value, which is constituted by ATP, adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), HxR and Hx (Zhao et al., 2019). From this aspect, nucleotidases in spoilage bacteria are strongly associated with fish freshness. The activity of nucleotidases in strain ABa4 increased by

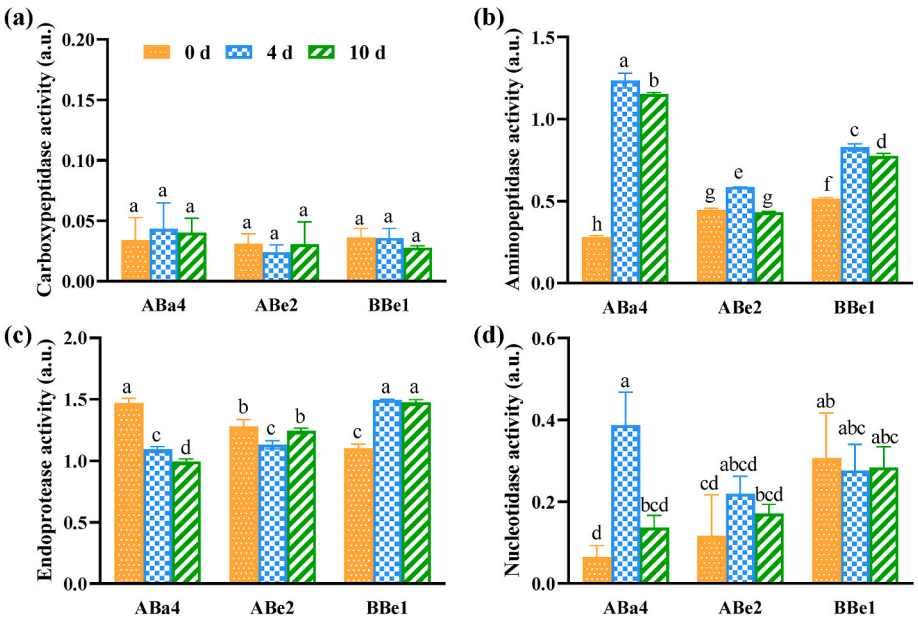

Fig. 2. Changes in activities of carboxypeptidase (a), aminopeptidase (b), acid proteinase (c) and nucleotidase (d) in *S. baltica* during spoilage. Note: Different letters indicate a significant difference ($P < 0.05$).

approximately 5-fold during 4 d storage but significantly decreased after 10 d storage, suggesting that strain ABa4 might mainly degrade nucleotides during early spoilage stage. The high level of nucleotidase activity of strain BBe1 indicated that strain BBe1 was active in consuming nucleotides during the whole spoilage period.

3.3. Metabolic profiles of *S. baltica* in fish broth during spoilage

The representative ^{1}H NMR spectra of metabolites from strain ABa4 in fish broths on days 0, 4 and 10 are shown in Fig. S2. The summary of identified metabolites in *S. baltica* is presented in Table S3, including amino acids, organic acids, sugars, alcohols, and nucleotide related compounds.

After the quantification with non-overlapping chemical shift peaks, a heatmap on a blue-red scale was plotted (Fig. 3). In a row, a hotter colour means a higher abundance of this metabolite among different groups. Metabolites in three strains on day 0 presented in bluish colours and the colours were transited to red or orange after chilled storage, suggesting that the metabolisms of *S. baltica* were enhanced during spoilage. There are some differences in metabolite contents in different strains on the same storage day. In strain ABa4 and ABe2, metabolite contents on day 4 showed hot colours, while most of red colours for metabolites in strain BBe1 were on day 10. This indicated that strain ABa4 and ABe2 might have high metabolic activities in early stage of spoilage but strain BBe1 might present a higher activity in later stage.

To deeply elucidate overall metabolite changes of three *S. baltica* strains in fish broth during spoilage, PCA was performed and visualized (Fig. 4). The model quality parameter with 0.94 for R^2X and 0.88 for Q^2

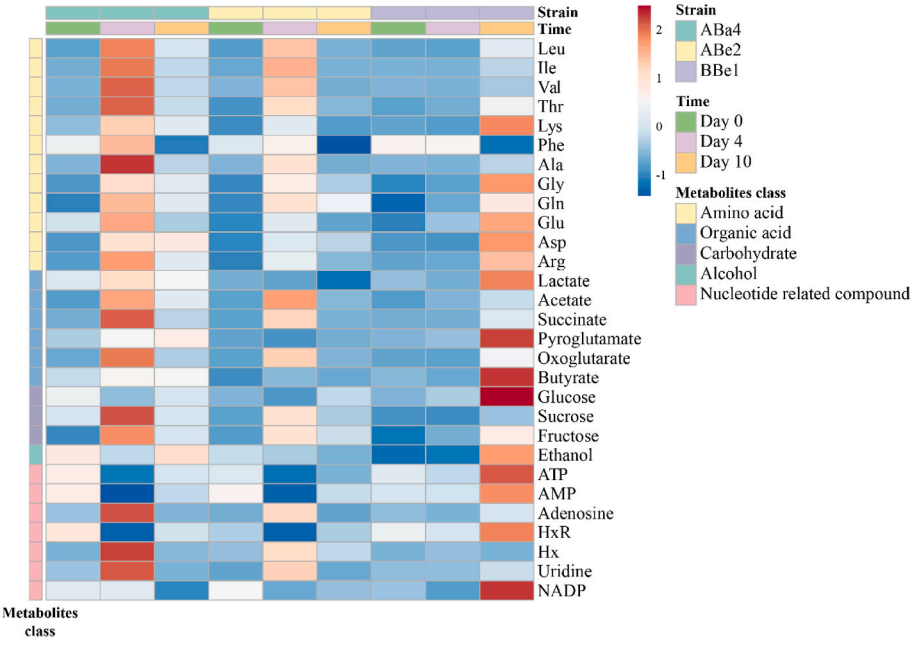

Fig. 3. Heatmap of metabolites in *S. baltica* strains during spoilage.

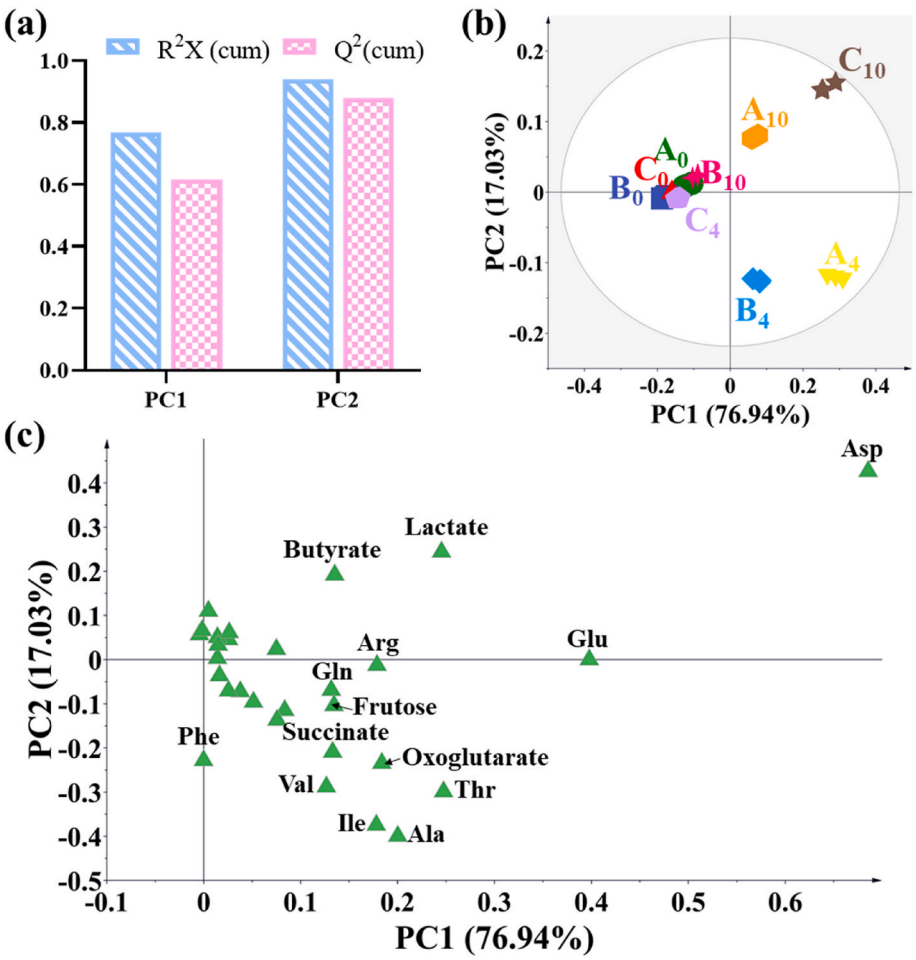

Fig. 4. Principal component analysis (PCA) of ^{1}NMR spectra of three *S. baltica* strains during spoilage. The variances are explained by principal components (a), the score plot (b) and the loading plot (c). Note: A_0, A_4, A_{10}: strain ABa4 on day 0, 4 and 10, respectively; B_0, B_4, B_{10}: strain ABe2 on day 0, 4 and 10, respectively; C_0, C_4, C_{10}: strain BBe1 on day 0, 4 and 10, respectively.

(Fig. 4a) indicated the good interpretation and prediction capability of this PCA model. The first two principal components (PCs) explained 93.97% (PC1: 76.94%, PC2: 17.03%) of the total variance (Fig. 4b). The metabolic profiles of three strains on day 0 located on the negative side of PC1. The profiles of strain ABa4 and ABe2 tended to the positive side of PC1 and negative side of PC2 during early stage and turned back after longer storage. On the contrary, the metabolic profiles of strain BBe1 only showed significant change during later spoilage. As shown in the loading plot (Fig. 4c), Asp and Glu highly contributed to PC1, and Asp, lactate and butyrate had large loadings on PC2.

3.4. Alternative metabolites in S. baltica during spoilage and pathway analysis

In order to reveal more information about the alternative metabolites of *S. baltica* during early and later spoilage, volcano plots were plotted and pathway analysis was performed (Figs. 5 and 6). The metabolites with |Fold change| > 1 and -Log_{10} P > 1.3 were regarded to be significantly changed. The pink points in volcano plots on the right side meant the increase of metabolites, while the metabolites with blue points indicated the decreased contents.

During the first 4 d storage, a distinct enhancement of amino acids, organic acids and sugars was observed in strain ABa4 and ABe2, accompanied with a decrease of ATP, AMP and HxR, while there was almost no significant change of metabolite content in strain BBe1 (Fig. 5). Ghaly et al. (2010) stated that bacterial activity was the major reason for fish spoilage when the storage time longer than 6 d, since the spoilage bacteria were still under proliferation phase during the initial stage of spoilage. Leyva-Díaz et al. (2017) mentioned that *S. baltica* could utilise a number of amino acids, organic acids and carbohydrates as carbon sources for growth and respiration. The translocases were found in *S. baltica* strains (Table S1), which could transport molecules from the outer membrane into the cell matrix. ATP, AMP and HxR were connected with energy metabolism, and could be converted to smaller nucleotide derivatives (Mao et al., 2013). In our study, the accumulated metabolites in strain ABa4 and ABe2 might be derived from fish broths and converted from other molecules. On the other hand, the insignificant metabolic changes in strain BBe1 might be due to the equilibrium between metabolite intake and consumption.

In the period from 4 d to 10 d, the contents of Leu, Thr, Ile, Val, Ala, Phe, acetate, oxoglutarate, succinate, sucrose, adenosine, Hx and uridine in strain ABa4 and ABe2 decreased, while all metabolites increased in strain BBe1 (Fig. 6), except Phe and Hx. Lou et al. (2021) demonstrated that the population of *S. baltica* in fish broths showed no significant difference when the storage time was longer than 8 d. This suggested that *S. baltica* in this study stepped into stationary phase around day 10 (Vine et al., 2004). Jaishankar and Srivastava (2017) stated stationary phase was the stage in which the bacterial growth rate and death rate were equal, but the metabolism of cells was still active. Hence, the decreased metabolites in strain ABa4 and ABe2 during later spoilage could be attributed to these metabolites being consumed via active metabolisms. Strain BBe1 showed enhanced levels of metabolites during later spoilage might be because the intake of small molecules from fish broths was greater than the consumption in this strain.

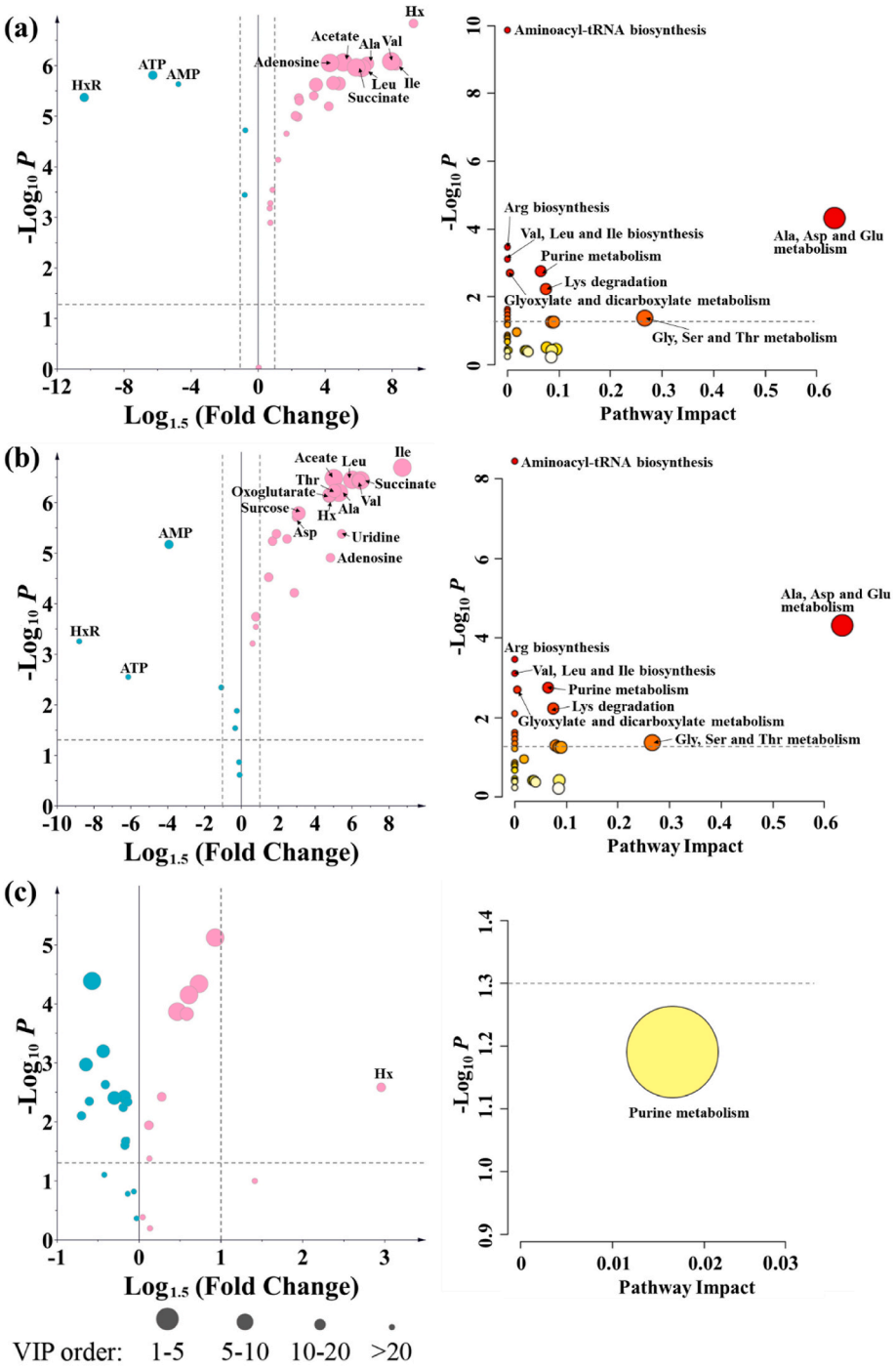

Fig. 5. Volcano plot (left) and pathway analysis (right) of the metabolite changes in strain ABa4 (a), strain ABe2 (b) and strain BBe1 (c) between day 0 and day 4. Note: Blue and pink colours in the volcano plot show the decreased and increased amount during spoilage, respectively.

Pathway analysis indicated that these altered metabolites during whole spoilage were related to aminoacyl-tRNA biosynthesis, Ala, Asp and Glu metabolism, Arg biosynthesis, Val, Leu and Ile biosynthesis, purine metabolism, glyoxylate and dicarboxylate metabolism, Lys degradation, nicotinate and nicotinamide metabolism, monobactam biosynthesis, Lys biosynthesis, Val, Leu and Ile degradation, Gly, Ser and Thr metabolism, cyanoamino acid metabolism, Tau and HyTau, glutathione metabolism, sulfur metabolism, tricarboxylic acid (TCA) cycle, Gln and Glu metabolism, nitrogen metabolism, and glycolysis (Figs. 5 and 6 and Tables S4 and S5). In the early stage, no pathway was perturbed in strain BBe1 but several amino acid metabolisms and organic acid metabolisms were affected in strain ABa4 and ABe2. Similar influenced pathways in strain ABa4 and ABe2 were observed during later spoilage. Apart from metabolisms shown in strain ABa4 and ABe2, Gln and Glu metabolism, nicotinate and nicotinamide metabolism, nitrogen metabolism, glycolysis, monobactam biosynthesis, Lys biosynthesis were significantly influenced in strain BBe1 in the period of later stage. Aminoacyl-tRNA biosynthesis was the most affected pathway in *S. baltica* during spoilage, with the 6–10 hit metabolites in three strains. In aminoacyl-tRNA biosynthesis, amino acids were carried by aminoacyl-tRNA in ribosomes for protein synthesis, thus influencing signal transduction and cell proliferation (Guo et al., 2021).

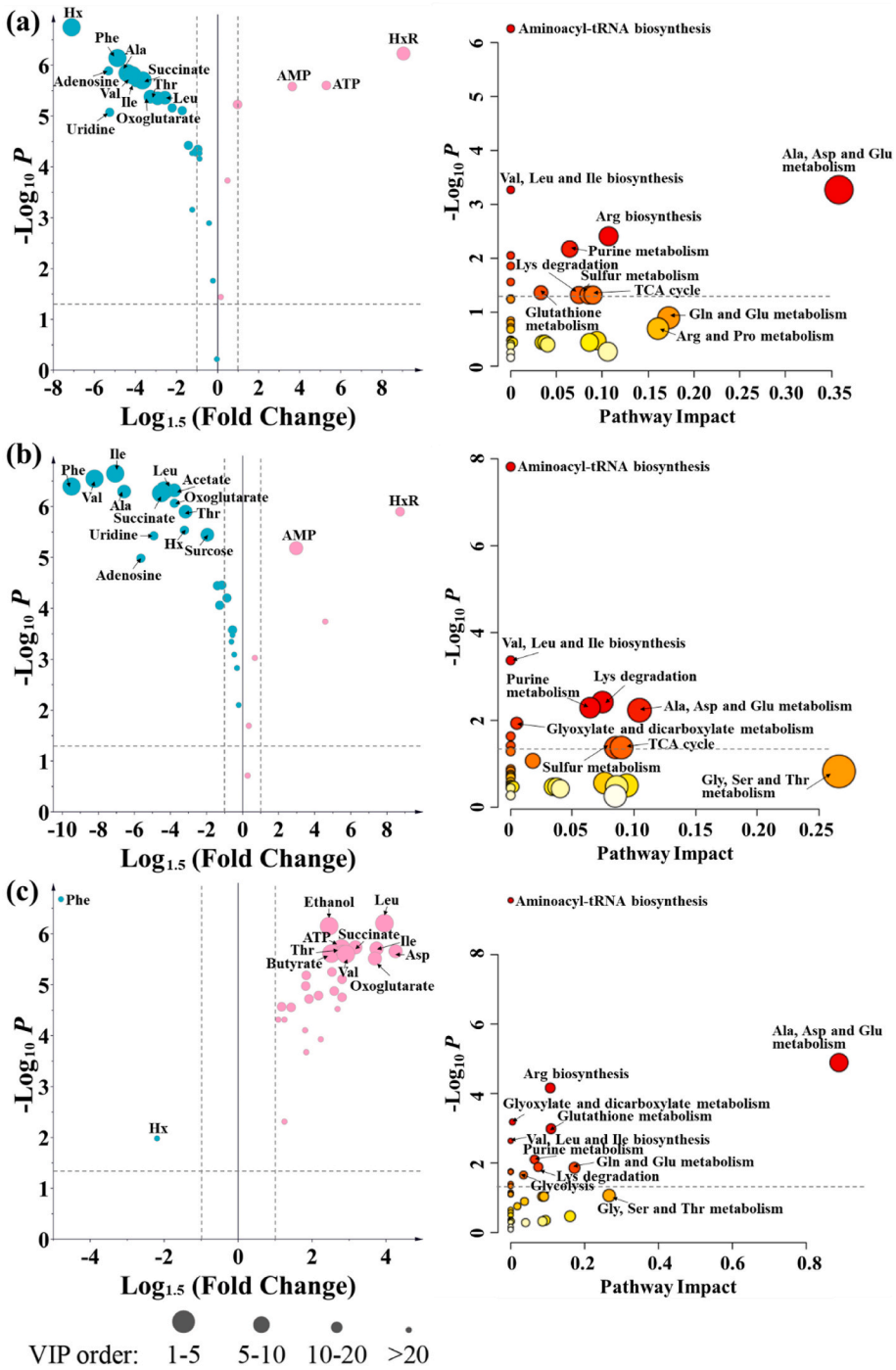

Fig. 6. Volcano plot (left) and pathway analysis (right) of the metabolite changes in strain ABa4 (a), strain ABe2 (b) and strain BBe1 (c) between day 4 and day 10. Note: Blue and pink colours in the volcano plot show the decreased and increased amount during spoilage, respectively.

López-Caballero et al. (2001) observed that agmatine, putrescine, spermidine and phenethylamine were produced by *S. putrefaciens* during incubation, which were related to Arg and Pro metabolism, and Phe metabolism. Lactate could be the H-donor for the transition from TMAO to TMA (Debevere et al., 2001), which might be regulated by TMAO reductase system protein (*tor*E), TMAO reductase (*tor*A), histidine kinase (*tor*S), periplasmic protein (*tor*T), and response regulator (*tor*R) in *S. baltica* (Li et al., 2020). Besides amino acid metabolism, sulfur metabolism was reported to contribute to H_2S production, and was connected with adenylyl-sulfate kinase (*cys*C) and phosphoadenylyl-sulfate reductase (*cys*H) (Yi & Xie, 2022).

3.5. Schematic illustration

Based on the results of enzyme activities, multivariate data analysis and pathway analysis, an assumptive schematic showing the enzymatic and metabolic changes of *S. baltica* during spoilage is proposed in Fig. 7. In the early stage of spoilage, *S. baltica* was in exponential phase (Lou et al., 2021; Vine et al., 2004), and nutrients from fish broths were taken for bacterial proliferation and activity. Amino acids, organic acids and sugars were transferred into the cell matrix in this period, together with the production of aminopeptidases, endoproteases and nucleotidases by *S. baltica*. In the later stage of spoilage, proteases and nucleotidases were still produced while more of them were secreted out of the cell (Chróst,

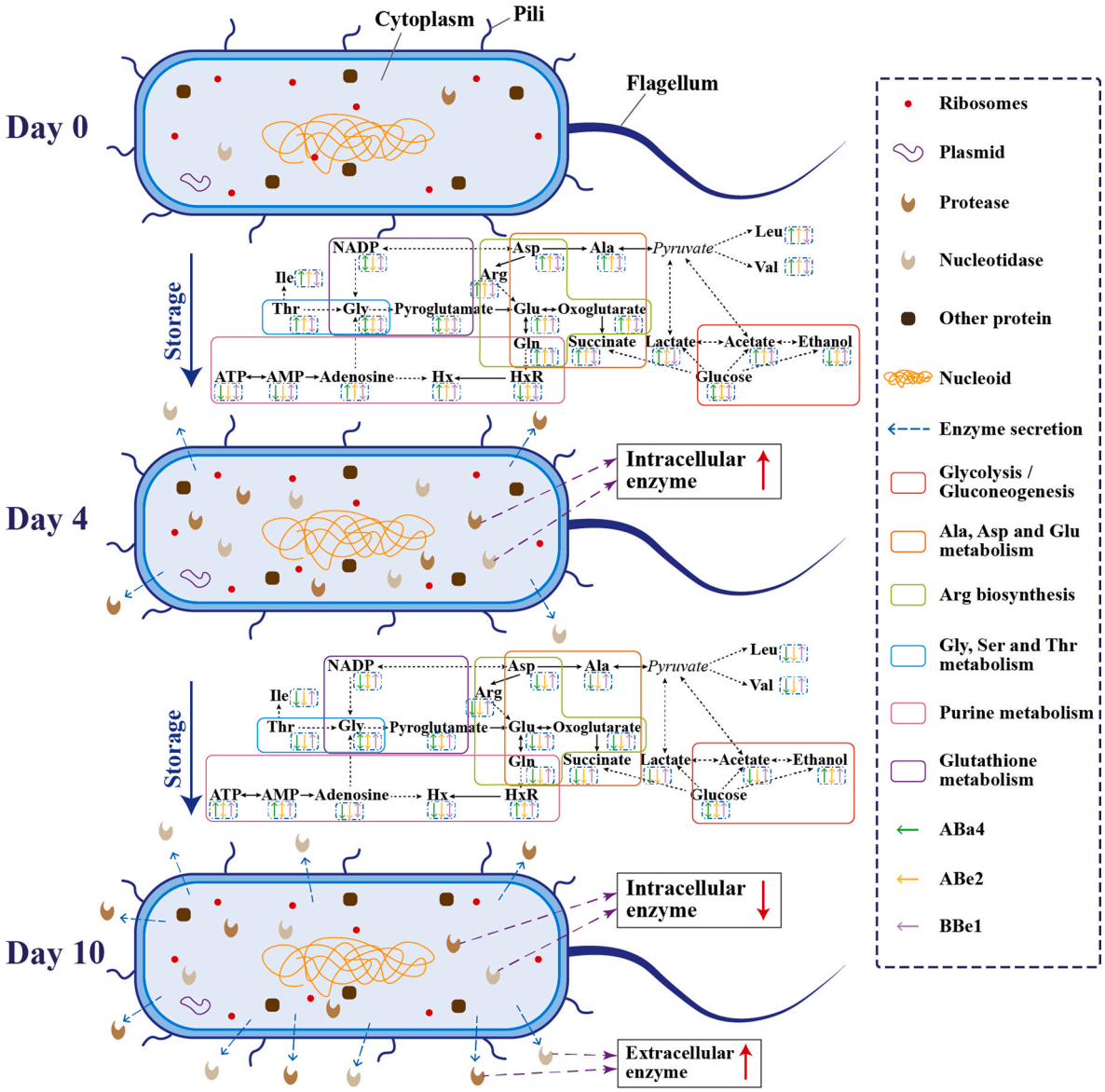

Fig. 7. Schematic of metabolic alterations in three *S. baltica* strains during spoilage. Note: Metabolite in an italic font is not identified; Upward, downward or double-headed arrow beside the metabolites represents a higher, lower or similar level after storage, respectively; transformation with dotted or solid line means that this reaction has or does not have intermediate product(s).

1991; Ge et al., 2017). The growth and metabolism of microorganisms is the major cause of fish spoilage in this stage (Ghaly et al., 2010), which is closely related to these extracellular enzymes generated by spoilage bacteria (Lou et al., 2021; Venugopal, 1990). *S. baltica* was in stationary phase and the metabolites inside bacteria were consumed.

4. Conclusion

In conclusion, the main enzymes of *S. baltica*, and enzyme activities as well as changes of intracellular metabolites of *S. baltica* in fish broth during spoilage, were examined. During early spoilage, proteases and nucleotidases were accumulated in strains, and metabolites were transferred into *S. baltica* for proliferation and activity. During later spoilage, more enzymes were secreted out of the cell, accompanied by the metabolite consumption in strain ABa4 and ABe2. Strain BBe1 showed a lag of metabolite change, compared to other strains. Amino acid metabolisms, aminoacyl-tRNA biosynthesis and purine metabolism were the main altered pathways about the spoilage activity of *S. baltica*. Overall, this study explores the spoilage mechanism of *S. baltica*, and contributes to theoretical guidance for seafood preservation.

CRediT authorship contribution statement

Xiaowei Lou: Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Visualization, Writing – original draft. **Yaowen Hai:** Data curation, Formal analysis, Investigation. **Yi Le:** Resources, Validation. **Xinli Ran:** Software, Data curation. **Hongshun Yang:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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.

Acknowledgements

This study was supported by Applied Basic Research Project (Agricultural) Suzhou Science and Technology Planning Programme (SNG2020061), an industry grant from Shanghai ProfLeader Biotech Co., Ltd (R-160-000-A21-597), and Singapore Ministry of Education Academic Research Fund Tier 1 (A-8000469-00-00).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2022.109341.

References

Ahangari, H., Yazdani, P., Ebrahimi, V., Soofiyani, S. R., Azargun, R., Tarhriz, V., & Eyvazi, S. (2021). An updated review on production of food derived bioactive peptides; focus on the psychrotrophic bacterial proteases. *Biocatalysis and Agricultural Biotechnology, 35*, Article 102051. https://doi.org/10.1016/j.bcab.2021.102051

Alfaro, B., Hernández, I., Le Marc, Y., & Pin, C. (2013). Modelling the effect of the temperature and carbon dioxide on the growth of spoilage bacteria in packed fish products. *Food Control, 29*(2), 429–437. https://doi.org/10.1016/j.foodcont.2012.05.046

AVA. (2021). *AVA annual report 2020/21*. https://www.sfa.gov.sg/docs/default-source/publication/annual-report/sfa-ar-2020-20212c7b8b52e3e84fd193c56d53f42fe607.pdf.

Boziaris, I. S., & Parlapani, F. F. (2017). Specific spoilage organisms (SSOs) in fish. In A. Bevilacqua, M. R. Corbo, M. Sinigaglia, & R. Sykes (Eds.), *The microbiological quality of food: Foodborne spoilers* (pp. 61–98). Woodhead Publishing. https://doi.org/10.1016/b978-0-08-100502-6.00006-6.

Chen, Z.-Q., & He, S.-H. (2004). Cloning and expression of human colon mast cell carboxypeptidase. *World Journal of Gastroenterology, 10*(3), 342–347. https://doi.org/10.3748/wjg.v10.i3.342

Chouliara, I., Savvaidis, I. N., Panagiotakis, N., & Kontominas, M. G. (2004). Preservation of salted, vacuum-packaged, refrigerated sea bream (*Sparus aurata*) fillets by irradiation: Microbiological, chemical and sensory attributes. *Food Microbiology, 21*(3), 351–359. https://doi.org/10.1016/S0740-0020(03)00065-0

Chróst, R. J. (1991). Environmental control of the synthesis and activity of aquatic microbial ectoenzymes. In R. J. Chróst (Ed.), *Microbial enzymes in aquatic environments* (pp. 29–59). Springer New York. https://doi.org/10.1007/978-1-4612-3090-8_3.

Debevere, J., Devlieghere, F., Van Sprundel, P., & De Meulenaer, B. (2001). Influence of acetate and CO_2 on the TMAO-reduction reaction by *Shewanella baltica*. *International Journal of Food Microbiology, 68*(1–2), 115–123. https://doi.org/10.1016/S0168-1605(01)00484-6

FAO. (2020). The state of world fisheries and aquaculture 2020. *Sustainability in action*. https://www.fao.org/3/ca9229en/ca9229en.pdf.

Feng, L., Bi, W., Chen, S., Zhu, J., & Liu, X. (2021). Regulatory function of sigma factors RpoS/RpoN in adaptation and spoilage potential of *Shewanella baltica*. *Food Microbiology, 97*, Article 103755. https://doi.org/10.1016/j.fm.2021.103755

Feng, X., Zhu, Y., Liu, Q., Lai, S., & Yang, H. (2017). Effects of bromelain tenderisation on myofibrillar proteins, texture and flavour of fish balls prepared from golden pomfret. *Food and Bioprocess Technology, 10*(10), 1918–1930. https://doi.org/10.1007/s11947-017-1963-7

Fu, L., Wang, C., Liu, N., Ma, A., & Wang, Y. (2018). Quorum sensing system-regulated genes affect the spoilage potential of *Shewanella baltica*. *Food Research International, 107*, 1–9. https://doi.org/10.1016/j.foodres.2018.01.067

Ge, Y., Zhu, J., Ye, X., & Yang, Y. (2017). Spoilage potential characterization of *Shewanella* and *Pseudomonas* isolated from spoiled large yellow croaker (*Pseudosciaena crocea*). *Letters in Applied Microbiology, 64*(1), 86–93. https://doi-org.libproxy1.nus.edu.sg/10.1111/lam.12687.

Ghaly, A. E., Dave, D., Budge, S., & Brooks, M. (2010). Fish spoilage mechanisms and preservation techniques: Review. *American Journal of Applied Sciences, 7*(7), 859–877. https://doi.org/10.3844/ajassp.2010.859.877

Gram, L., & Dalgaard, P. (2002). Fish spoilage bacteria-problems and solutions. *Current Opinion in Biotechnology, 13*(3), 262–266. https://doi.org/10.1016/S0958-1669(02)00309-9

Guo, F., Liang, Q., Zhang, M., Chen, W., Chen, H., Yun, Y., Zhong, Q., & Chen, W. (2021). Antibacterial activity and mechanism of linalool against *Shewanella putrefaciens*. *Molecules, 26*(1), 245. https://www.mdpi.com/1420-3049/26/1/245.

Jaishankar, J., & Srivastava, P. (2017). Molecular basis of stationary phase survival and applications. *Frontiers in Microbiology, 8*. https://doi.org/10.3389/fmicb.2017.02000, 2000.

Kim, M. J., Kim, H. J., Kim, K. H., Heu, M. S., Lee, J. S., & Kim, J.-S. (2012). Fractionation and enzymatic characterization of endoprotease and exopeptidase from crude extracts of cuttlefish Sepia officinalis hepatopancreas. *Fisheries and Aquatic Sciences, 15*(4), 283–291. https://doi.org/10.5657/FAS.2012.0283

Leyva-Díaz, J. C., Poyatos, J. M., Barghini, P., Gorrasi, S., & Fenice, M. (2017). Kinetic modeling of *Shewanella baltica* KB30 growth on different substrates through respirometry. *Microbial Cell Factories, 16*(1), 189. https://doi.org/10.1186/s12934-017-0805-7

Li, J., Yu, H., Yang, X., Dong, R., Liu, Z., & Zeng, M. (2020). Complete genome sequence provides insights into the quorum sensing-related spoilage potential of *Shewanella baltica* 128 isolated from spoiled shrimp. *Genomics, 112*(1), 736–748. https://doi.org/10.1016/j.ygeno.2019.05.010

Loo, L. S. W., Vethe, H., Soetedjo, A. A. P., Paulo, J. A., Jasmen, J., Jackson, N., Bjørlykke, Y., Valdez, I. A., Vaudel, M., Barsnes, H., Gygi, S. P., Ræder, H., Teo, A. K. K., & Kulkarni, R. N. (2020). Dynamic proteome profiling of human pluripotent stem cell-derived pancreatic progenitors. *Stem Cells, 38*(4), 542–555. https://doi.org/10.1002/stem.3135

López-Caballero, M. E., Sánchez-Fernández, J. A., & Moral, A. (2001). Growth and metabolic activity of Shewanella putrefaciens maintained under different CO2 and O2 concentrations. *International Journal of Food Microbiology, 64*(3), 277–287. https://doi.org/10.1016/S0168-1605(00)00473-6

Lou, X., Zhai, D., & Yang, H. (2021). Changes of metabolite profiles of fish models inoculated with *Shewanella baltica* during spoilage. *Food Control, 123*, Article 107697. https://doi.org/10.1016/j.foodcont.2020.107697

Macé, S., Joffraud, J.-J., Cardinal, M., Malcheva, M., Cornet, J., Lalanne, V., Chevalier, F., Sérot, T., Pilet, M.-F., & Dousset, X. (2013). Evaluation of the spoilage potential of bacteria isolated from spoiled raw salmon (*Salmo salar*) fillets stored under modified atmosphere packaging. *International Journal of Food Microbiology, 160*(3), 227–238. https://doi.org/10.1016/j.ijfoodmicro.2012.10.013

Mao, S., Fu, G., Seese, R. R., & Wang, Z.-Y. (2013). Estimation of PMI depends on the changes in ATP and its degradation products. *Legal Medicine, 15*(5), 235–238. https://doi.org/10.1016/j.legalmed.2013.03.004

Mol, S., Erkan, N., Uecok, D., & Tosun, Ş. Y. (2007). Effect of psychrophilic bacteria to estimate fish quality. *Journal of Muscle Foods, 18*(1), 120–128.

Parlapani, F. F., Mallouchos, A., Haroutounian, S. A., & Boziaris, I. S. (2017). Volatile organic compounds of microbial and non-microbial origin produced on model fish substrate un-inoculated and inoculated with gilt-head sea bream spoilage bacteria. *LWT–Food Science and Technology, 78*, 54–62. https://doi.org/10.1016/j.lwt.2016.12.020

Prabhakar, P. K., Vatsa, S., Srivastav, P. P., & Pathak, S. S. (2020). A comprehensive review on freshness of fish and assessment: Analytical methods and recent innovations. *Food Research International, 133*, Article 109157. https://doi.org/10.1016/j.foodres.2020.109157

Sales, P. B. V., & Santoro, M. L. (2008). Nucleotidase and DNase activities in Brazilian snake venoms. *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology, 147*(1), 85–95. https://doi.org/10.1016/j.cbpc.2007.08.003

Sinsuwan, S., Rodtong, S., & Yongsawatdigul, J. (2008). Characterization of Ca^{2+}-activated cell-bound proteinase from *Virgibacillus* sp. SK37 isolated from fish sauce fermentation. *LWT - Food Science and Technology, 41*(10), 2166–2174. https://doi.org/10.1016/j.lwt.2008.02.002

Venugopal, V. (1990). Extracellular proteases of contaminant bacteria in fish spoilage: A review. *Journal of Food Protection, 53*(4), 341–350. https://doi.org/10.4315/0362-028x53.4.341

Vine, N. G., Leukes, W. D., & Kaiser, H. (2004). In vitro growth characteristics of five candidate aquaculture probiotics and two fish pathogens grown in fish intestinal mucus. *FEMS Microbiology Letters, 231*(1), 145–152. https://doi.org/10.1016/s0378-1097(03)00954-6

Wang, Y., Wu, J.e., & Yang, H. (2022). Comparison of the metabolic responses of eight *Escherichia coli* strains including the "big six" in pea sprouts to low concentration electrolysed water by NMR spectroscopy. *Food Control, 131*, Article 108458. https://doi.org/10.1016/j.foodcont.2021.108458

Wu, G., Wan, F., Fu, H., Li, N., Gao, H., & Metcalf, W. W. (2015). A matter of timing: Contrasting effects of hydrogen sulfide on oxidative stress response in *Shewanella oneidensis*. *Journal of Bacteriology, 197*(22), 3563–3572. https://doi.org/doi:10.1128/JB.00603-15.

Yi, Z., & Xie, J. (2022). Genomic analysis of two representative strains of *Shewanella putrefaciens* isolated from bigeye tuna: Biofilm and spoilage-associated behavior. *Foods, 11*(9), 1261. https://www.mdpi.com/2304-8158/11/9/1261.

Zhao, X., Wu, J.e., Chen, L., & Yang, H. (2019). Effect of vacuum impregnated fish gelatin and grape seed extract on metabolite profiles of tilapia (*Oreochromis niloticus*) fillets during storage. *Food Chemistry, 293*, 418–428. https://doi.org/10.1016/j.foodchem.2019.05.001

Zhu, J., Huang, X., Zhang, F., Feng, L., & Li, J. (2015). Inhibition of quorum sensing, biofilm, and spoilage potential in *Shewanella baltica* by green tea polyphenols. *Journal of Microbiology, 53*(12), 829–836. https://doi.org/10.1007/s12275-015-5123-3

Zhu, J., Yan, Y., Wang, Y., & Qu, D. (2019). Competitive interaction on dual-species biofilm formation by spoilage bacteria, *Shewanella baltica* and *Pseudomonas fluorescens*. *Journal of Applied Microbiology, 126*(4), 1175–1186. https://doi.org/10.1111/jam.14187

Zhu, J., Zhao, A., Feng, L., & Gao, H. (2016). Quorum sensing signals affect spoilage of refrigerated large yellow croaker (*Pseudosciaena crocea*) by *Shewanella baltica*. *International Journal of Food Microbiology, 217*, 146–155. https://doi.org/10.1016/j.ijfoodmicro.2015.10.020