Food Control 123 (2021) 107697

Contents lists available at ScienceDirect

Food Control

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

Changes of metabolite profiles of fish models inoculated with *Shewanella baltica* during spoilage

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ARTICLE INFO

Keywords:
Fish
NMR
Metabolite
Omics
Spoilage
Metabolomics
Seafood
Shewanella baltica
Microbial safety
Storage
Preservation
Pathway
Fish stick
Broth
Foodomics
Food safety
Amino acid
Biogenic amine
Nucleotide

ABSTRACT

Three *Shewanella baltica* strains (ABa4, ABe2, BBe1) were inoculated into sterile fish sticks and broths, and the metabolic changes during chilled storage were determined using NMR. Totally 39 metabolites in two fish models were identified, which were involved in four main metabolic pathways: peptide and amino acid, nitrogen, nucleotide, and carbohydrate pathways. In fish sticks, proteins were hydrolysed to increase amino acids (up to 230%) by *S. baltica*, especially by strain ABa4. In both fish sticks and broths, *S. baltica* induced the formations of biogenic amines from amino acids and trimethylamine-N-oxide (TMAO), and the degradations of adenine nucleotides to form inosine and hypoxanthine (2- to 4-fold increment). In addition, sugars and lactate were consumed by *S. baltica*, accompanied with the productions of acetate and succinate. Strain BBe1 showed higher TVB-N and capability of biogenic amine production and nucleotide degradation. The changes could be attributed to the enzymes of *S. baltica* during growth and respiration. These findings confirm that *S. baltica* has highly spoilage potential to decompose nutrients in fish, mainly via nitrogen and nucleotide pathways. This study provides insight on controlling seafood spoilage by intervening degradation pathways.

1. Introduction

Fish is reported as a rich source of high-quality proteins, lipids and essential micronutrients, which are beneficial to immune system, cardiovascular function and metabolic processes (Oz & Kotan, 2016). It is a popular food in Singapore, but only limited supply in local farms. To improve food security in Singapore, golden pomfret (*Trachinotus blochii*) was successfully spawned by Agri-Food & Veterinary Authority of Singapore (AVA) (Feng, Zhu, Liu, Lai, & Yang, 2017).

However, fish is highly perishable during storage due to endogenous or exogenous enzymes and microorganisms (Briones, Reyes, Tabilo-Munizaga, & Pérez-Won, 2010; Zeng, Liao, Terhune, & Wang, 2019). It has been reported that the main cause of fish putrefaction and deterioration above freezing temperature is microbial activity (Gennari, Tomaselli, & Cotrona, 1999; Parlapani, Mallouchos, Haroutounian, & Boziaris, 2017). The microbiota of fish at the initiation of marketing is composed of the natural bacteria which exist in digestive tract, skin and gills, and the bacterial populations that are contaminated from environment, workers, etc (Boziaris & Parlapani, 2017). Many studies reported spoilage bacteria in seafood, amongst which *Shewanella* spp. are specific spoilage organisms (SSOs) in low-temperature stored fish (Begoña Alfaro, Hernández, Le Marc, & Pin, 2013; Ghaly, Dave, Budge,

Abbreviations: NMR, nuclear magnetic resonance; SSOs, specific spoilage organisms; TMAO, trimethylamine-N-oxide; TMA, trimethylamine; TVB-N, total volatile basic nitrogen; TVC, total viable count; D_2O, deuterium oxide; LB, Luria-Bertani; TSP, sodium 3-trimethylsilyl [2,2,3,3-d_4] propionate; 1D, one-dimensional; 2D, two-dimensional; HSQC, heteronuclear single quantum correlation; PCA, principal component analysis; OPLS-DA, orthogonal projection to latent structure with discriminant analysis; ATP, adenosine triphosphate; IMP, inosine monophosphate; AMP, adenosine monophosphate; DMA, dimethylamine.

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https://doi.org/10.1016/j.foodcont.2020.107697
Received 28 June 2020; Received in revised form 4 October 2020; Accepted 12 October 2020
Available online 16 October 2020

& Brooks, 2010).

Shewanella spp. are a group of Gram-negative, facultative anaerobic, H_2S-producing rods, which have been frequently separated from spoiling seafood and marine water (Zhu, Huang, Zhang, Feng, & Li, 2015). They play a crucial role in the transformation from trimethylamine-N-oxide (TMAO) to trimethylamine (TMA), causing a "fishy" off-odour (Debevere, Devlieghere, Van Sprundel, & De Meulenaer, 2001; Zhu, Huang, et al., 2015).

Shewanella baltica is a species reclassified from *Shewanella putrefaciens* (Ziemke, Höfle, Lalucat, & Rossellö-Mora, 1998). Several studies have reported isolated *S. baltica* from spoiled fish and its spoilage potential. Alfaro and Hernandez (2013) identified *S. baltica* as one of the dominant phylotypes in Atlantic horse mackerel (*Trachurus trachurus*) after 5 d storage. Gu, Fu, Wang, and Lin (2013) showed that 48 strains (out of 102 spoilage strains) were identified as *S. baltica* from large yellow croaker (*Pseudosciaena crocea*) at 4 °C storage. Macé et al. (2013) reported that *S. baltica* produced a mix of spoilage flavour (feet/cheese, sour and amines) in inoculated salmon (*Salmo salar*) fillets. In addition, Zhu, Zhao, Feng, and Gao (2016) observed different increments of TMA, total volatile basic nitrogen (TVB-N) and putrescine in fish broths inoculated with different strains of *S. baltica* during storage.

Sterile fish stick and broth are useful fish models for microbial spoilage in seafood research (Gu et al., 2013; Liu et al., 2018). Sterile fish stick is prepared by surface sterilisation, skin removal and ultraviolet treatment, while sterile fish broth is prepared by homogenisation with water, boiling, filtration and autoclaving. Sterile fish stick and broth can provide different amounts of nutrients and create different environments to microorganisms, so spoilage bacteria might show different activities in them (Lerke, Adams, & Farber, 1963). Up to now, little information is available on the effect of different strains of *S. baltica* on metabolites in different sterile fish models.

It has been reported that nuclear magnetic resonance (NMR) is capable of providing comprehensive information of low molecular weight compounds in food (Chen, Ye, Chen, Zhan, & Lou, 2017; Lou et al., 2018). It is also a powerful technique for analysing the metabolic activities of bacteria *in vitro* (Chen et al., 2020; Liu et al., 2017). Moreover, some metabolites identified from NMR spectroscopy are regarded as the freshness indicators of seafood, which shows that seafood quality can be reflected using NMR-based metabolic characterisation (Shumilina, Ciampa, Capozzi, Rustad, & Dikiy, 2015).

In this study, different strains of *S. baltica* were inoculated into two sterile fish models. The changes of metabolite profiles of fish sticks and broths during storage induced by *S. baltica* were analysed using NMR. TVB-N and total viable count (TVC) of *S. baltica* were determined to further certify the metabolic results. The objectives were to investigate the spoilage mechanism of *S. baltica* in fish, and to provide information for controlling seafood spoilage during chilled storage.

2. Materials and methods

2.1. Chemicals and reagents

Deuterium oxide (D_2O, 99.9%) was purchased from Cambridge Isotope Laboratories (Miami, FL, USA). Other chemicals were analytical grade, and purchased from Sigma-Aldrich (St. Louis, MO, USA). Agar and broth were purchased from Oxoid (UK).

2.2. Preparation of sterile fish sticks and broths

Golden pomfret (*Trachinotus blochii*) with average bodyweight of 526 ± 30 g were purchased from a local supermarket (Sheng Siong) in Singapore. Fish were gutted and transported to the laboratory within 1 h in an ice box.

Sterile fish sticks were prepared according to Liu et al. (2018) and Macé et al. (2013) with slight modification. The fish were cleaned with sterile distilled water without destroying skin and the membrane of gut cavity. The skin surface and gut cavity of fish were washed in 5% Na_2CO_3 twice, washed in 2% formalin solution, swabbed with 70% ethanol, and dried under the flow of sterile air, respectively. Portions of skin were removed aseptically and fish sticks were excised using sanitised knives and cutting boards. Ten grams of fish sticks were placed in a sterile Petri dish and exposed under ultraviolet for 30 min. The sticks were stored at 4 °C and used within 4 h.

Sterile fish broths were prepared according to Parlapani et al. (2017) and Gu et al. (2013) with slight modification. Fish fillets (100 g) were cut and homogenised with distilled water (100 mL). The broths were separated using double gauze and boiled at 90 °C for 5 min. After cooling, the broths were filtered, mixed with 0.10 M phosphate buffer, and adjusted to pH 6.50 with NaOH/HCl. Afterward, the broths were autoclaved and cooled. Sterile fish broths were stored at 4 °C and used within 4 h.

2.3. Bacterial strains and inoculation

Three isolates of *S. baltica* (ABa4, ABe2, and BBe1) were previously isolated from spoiled golden pomfret fillets. The cryopreserved strains were activated by two consecutive 6 d transfers at 4 °C in 10 mL Luria-Bertani (LB) broth and plated onto LB agar to obtain discrete colonies. Colonies were re-cultured in LB broth at 4 °C until the inocula reached a final level of 6 log CFU/mL.

Sterile fish sticks were immerged into inocula and the final cell concentrations on sticks reached around 4 log CFU/g. The inoculated sticks were dried under the flow of sterile air for 5 min (Yemmireddy, Cason, Moreira, & Adhikari, 2020). Sterile fish broths were inoculated with inocula and the final cell concentrations in broths reached around 4 log CFU/mL. The inoculated fish sticks and broths were stored at 4 °C for 10 d. Metabolites of fish models were extracted and tested on day 0, 4 and 10. The populations of *S. baltica* and TVB-N were tested every two days.

2.4. Preparation of metabolites

Metabolites extraction in fish sticks were done according to Lou et al. (2018) and Zhao, Wu, Chen, and Yang (2019). Samples (extracts of 0.4 g of sticks or 0.4 mL of broths) were lyophilised and stored at 4 °C until using.

Each sample was dissolved in 600 μL deuterated water (D_2O, 99.9%) containing 0.01% sodium 3-trimethylsilyl [2,2,3,3-d_4] propionate (TSP) (Zhao, Zhao, Wu, Lou, & Yang, 2019). After centrifugation at 12, 000×*g* for 10 min at 4 °C, 550 μL supernatant was transferred into a 5 mm NMR tube.

2.5. NMR analysis

One-dimensional (1D) ^1H and two-dimensional (2D) NMR spectra were acquired according to our previous work with slight modification (Chen et al., 2019; Zhao, Wu, et al., 2019). ^1H NMR spectra of extracts from fish sticks and broths were performed at 25 °C on a Bruker DRX-500 NMR Spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a Triple Inverse Gradient probe operating at 500.23 MHz. Relaxation delay was 2 s; mixing time was 20 ms; scan was 128 times; spectral width was 20 ppm; acquisition time was 3.3 s. A 90° pulse length of each result was modified by an automatic pulse calculation in TopSpin 4.0.7 (Bruker Biospin, Rheinstetten, Germany). To identify the signal of metabolites, the 2D ^1H–^{13}C heteronuclear single quantum correlation (HSQC) was acquired at 25 °C. The width of ^1H spectra was 10.0 ppm and the width of the ^{13}C spectra was 165.0 ppm.

2.6. Spectral processing

The 1D ^1H and 2D NMR spectra were analysed using TopSpin 4.0.7 software (Bruker Biospin, Rheinstetten, Germany). The individual

metabolite was assigned using 1D and 2D spectra cooperatively according to related references and metabolome database of The Metabolomics Innovation Centre (http://www.hmdb.ca/).

After the normalisation of phases, baselines and TSP signal locations, the NMR data were processed using multivariate data analysis (SIMCA-P+, Umetrics, Sweden) and MATLAB R2019b (MathWorks, Natick, MA, USA) according to our previous work (Zhao, Chen, Wu, He, & Yang, 2020). Results were shown as principal component analysis (PCA) plots, orthogonal projection to latent structure with discriminant analysis (OPLS-DA) score plots and coefficient-coded loading plots. For quantitative analysis, the non-overlapping signals of metabolite and internal reference (TSP) were integrated in TopSpin 4.0.7 software. Metabolite concentration was calculated by equating the integrals of metabolite in relation to that of TSP.

2.7. Determination of TVB-N

TVB-N was determined according to Zhao, Wu, et al. (2019). Supernatant (5 mL) extracted from sticks or filtered broths (5 mL) were used for distillation.

For fish sticks: TVB-N (mg/100 g) $= \frac{(V_1-V_0)\times C\times 14\times V_h}{m\times 5}\times 100$

For fish broths: TVB-N (mg/100 mL) $= \frac{(V_1-V_0)\times C\times 14}{5}\times 100$Where V_1 and V_0 are the titrated volume (mL) for the sample and the blank, respectively; C is the concentration (M) of HCl; m is the sample mass (g); V_h is the volume (mL) of distilled water added to fish stick.

2.8. Enumeration of S. baltica

Ten grams of fish sticks were homogenised with 90 mL 0.1% peptone water using a Stomacher (Masticator Stomacher, IUL Instruments, Germany) for 90 s. Serial dilutions (1/10, v/v) for supernatant of each stick sample or broth sample were made (Zhao, Chen, Zhao, He, & Yang, 2020). TVC of *S. baltica* was determined on LB agar after 6 d incubation at 4 °C and expressed as log CFU/g for fish sticks or log CFU/mL for fish broths. Enrichment tests were applied for control fish stick and broth groups. The detection limits of control fish stick and broth were 1 log CFU/g and 0 log CFU/mL, respectively, and 2 log CFU/g and 1 log CFU/mL, respectively for treated groups.

2.9. Statistical analysis

All assays were done in triplicate independently. The changes of metabolite concentrations, TVB-N value and TVC of fish models during spoilage were analysed by two-way ANOVA (Duncan's Multiple Range Test) using software SAS 8.0 (SAS institute Inc. Cary, NC, USA). The significance of difference was $P < 0.05$.

3. Results and discussion

3.1. 1H NMR spectra of fish models

Fig. 1 shows representative ^{1}H NMR spectra of extracts from sterile fish sticks and broths inoculated with strain ABa4 on day 0, 4 and 10. Totally, 39 metabolites were identified and presented in Table S1, including 16 amino acids (Leu, Ile, etc), 1 dipeptide (anserine), 4 organic acids (lactate, acetate, etc), 3 sugars (α-glucose, β-glucose, etc), 1 alcohol (2,3-butanediol), 5 biogenic amines (putrescine, tyramine, etc), 6 nucleotides and their derivatives (adenosine, inosine, etc) and 3 other components (choline, betaine, etc). Most of them were reported in tilapia, salmon and pike eel (Chen et al., 2017; Shumilina et al., 2015; Zhao, Wu, et al., 2019). It was obvious that the intensities of some signals (such as lactate, inosine and hypoxanthine) changed from day 0 to day 10.

3.2. Principal components analysis

PCA scores plots of fish sticks and broths inoculated with *S. baltica* are shown in Fig. 2. PC1 and PC2 explained 95.2% of the total variance in fish sticks and 78.86% in fish broths. The uninoculated sticks on day 0, 4 and 10 were located in the positive side of PC1 and PC2, while the inoculated sticks on day 4 and 10 respectively located in the negative side of PC2 and PC1 (Fig. 2a). The metabolite profiles of inoculated broths tended to the negative sides of PC1, compared to the controls, which were located in the positive side of PC1 (Fig. 2b). In both fish models, the profiles of inoculated samples were significantly deviated from the controls after storage ($P < 0.05$), especially the samples on day 10, while there was no significant discrepancy of the profiles between samples inoculated with different strains on same day.

3.3. Alterative metabolites in fish models inoculated with S. baltica during spoilage

To further study the metabolic changes in fish models inoculated with *S. baltica* during storage, OPLS-DA cross-validated score plots and coefficient-coded loading plots were plotted. The values of R^2X and Q^2 of OPLS-DA are shown in Table S2, indicating significant intergroup differences and good model predictabilities. Figs. 3 and 4 show the pairwise comparative OPLS-DA for fish sticks and broths inoculated with strain ABa4, respectively. The OPLS-DA results of strain ABe2 and BBe1 (Figs. S1, S2, S3 and S4) were similar to those of strain ABa4 (Figs. 3 and 4). The correlation coefficients of comparisons of fish sticks and broths are shown in Tables S3 and S4, respectively. The metabolites with coefficients >0.602 were recognised to be significant in this study ($P < 0.05$).

The results revealed that *S. baltica* considerably changed the metabolites in fish models. The changes became more obvious with prolonged chilled storage. In fish sticks, *S. baltica* induced a distinct increase in the levels of inosine, hypoxanthine, acetate succinate, anserine, Asp, Val, Ile, Arg, Gln, Glu, Lys, Phe, Tyr, Ala, Leu, Met, tyramine, 2,3-butanediol, creatinine and betaine, accompanied with a decline of adenosine triphosphate (ATP), inosine monophosphate (IMP), adenosine monophosphate (AMP), adenosine, Gly, lactate, creatine, glucose and choline. In fish broths, the changes of organic acids, nucleotides and their derivatives were similar to those in sticks, but most amino acids decreased during storage.

The correlation coefficients (Tables S3 and S4) were conducted to show the significance of metabolites in paired groups and were in accordance with OPLS-DA coefficient-coded loading plots (Figs. 3, 4, S1, S2, S3 and S4). During 10 d chilled storage, 3 different *S. baltica* strains increased 23–24 metabolites in fish sticks and 5–7 metabolites in fish broths, but decreased 10–11 and 26–30 metabolites in fish sticks and broths, respectively. In terms of OPLS-DA results, the change of metabolite profile of strain ABa4 was most significant, especially in fish broth on day 4, indicating that strain ABa4 had higher metabolic ability than other strains during early spoilage.

3.4. Metabolite quantification analysis

The concentrations of metabolites in fish sticks and broths were quantified, as shown in Tables 1 and 2, respectively. Thr, γ-aminobutyric acid, putrescine and TMAO were not quantified due to the overlapped chemical shifts.

The contents of metabolites in inoculated fish sticks during 10 d storage increased ($P < 0.05$), expect for Gly, lactate, creatine, glucose, ATP, AMP, adenosine, IMP and choline, compared to the control. Such enhancements were highlighted with a 6.7-fold increment in acetate as well as 2.5-fold increment in hypoxanthine, inosine and dimethylamine (DMA). The contents of most amino acids increased by approximate 1- to 2-fold. On the contrary, the abundances of adenosine, lactate and IMP decreased by approximate half.

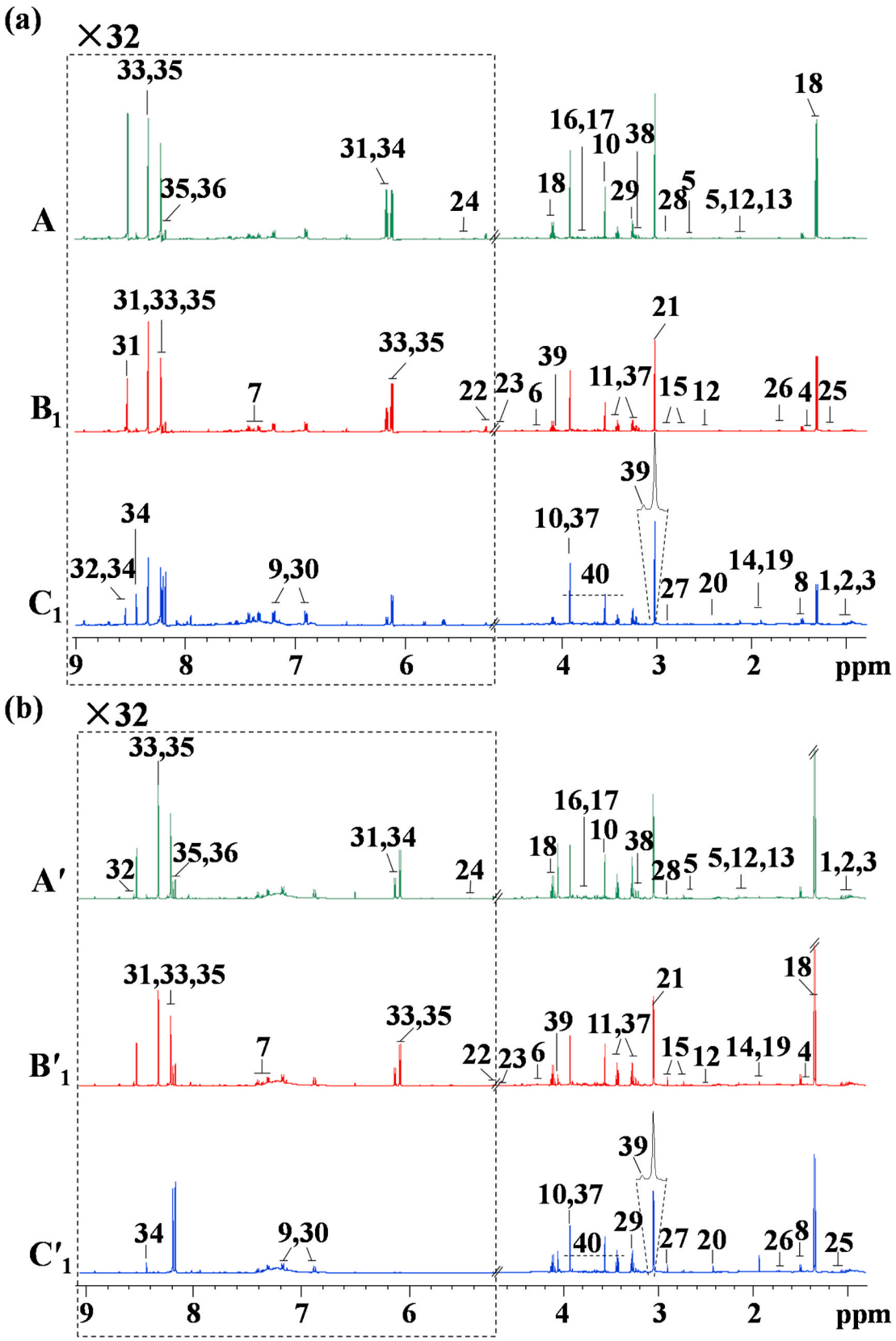

Fig. 1. Representative ^{1}H NMR spectra of fish sticks (a) and broths (b) inoculated with strain ABa4 on day 0 (A & A′, control), day 4 (B_1 & B'_1) and day 10 (C_1 & C'_1). Keys: 1: Leu; 2: Ile; 3: Val: 4: Lys; 5: Met; 6: Thr; 7: Phe; 8: Ala; 9: Tyr; 10: Gly; 11: Tau; 12: Gln; 13: Glu; 14: γ-aminobutyric acid; 15: Asp; 16: Arg; 17: Anserine; 18: Lactate; 19: Acetate; 20: Succinate; 21: Creatine; 22: α-glucose; 23: β-glucose; 24: Maltose; 25: 2,3-butanediol; 26: Putrescine; 27: TMA; 28: DMA; 29: TMAO; 30: Tyramine; 31: ATP; 32: AMP; 33: Adenosine; 34: IMP; 35: Inosine; 36: Hypoxanthine; 37: Betaine; 38: Choline; 39: Creatinine; 40: Glucose and amino acids.

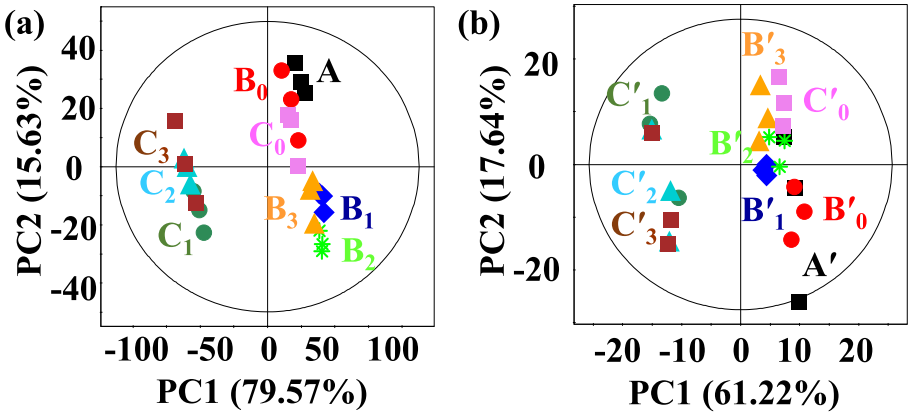

Fig. 2. PCA scores plots for metabolite profiles of fish sticks (a) and broths (b) inoculated with *S. baltica*. A & A′: uninoculated fish models on day 0; B_0 & B'_0: uninoculated fish models on day 4; B_1 & B'_1: fish models with strain ABa4 on day 4; B_2 & B'_2: fish models with strain ABe2 on day 4; B_3 & B'_3: fish models with strain BBe1 on day 4; C_0 & C'_0: uninoculated fish models on day 10; C_1 & C'_1: fish models with strain ABa4 on day 10; C_2 & C'_2: fish models with strain ABe2 on day 10; C_3 & C'_3: fish models with strain BBe1 on day 10.

The main quantitative difference of fish broths from sticks was the decreased amino acids ($P < 0.05$), amongst which Arg, Asp, Leu, Ala and Glu reduced most significantly (10–33% decrement). The reductions of ATP, IMP and adenosine in fish broths were higher than those of fish sticks, which were more than 90%. Compared to those in fish sticks, the elevations of succinate (10-fold), inosine (4-fold), hypoxanthine (4-fold) and TMA (2.5-fold) in fish broths were higher. The differences of metabolite contents between two fish models could be attributed to the different amounts of proteins, enzymes and precursors of metabolites in sticks and broths before storage. In addition, *S. baltica* might show different activities in fish sticks and broths, due to the liquid environment was liable to create an anaerobic environment (Lerke et al., 1963).

Shumilina et al. (2015) reported that the concentrations of free amino acids and organic acids increased but the contents of TMAO, IMP and sugars decreased in Atlantic salmon fillets during post-mortem storage. Tan, Huang, Feng, Li, and Cai (2018) demonstrated the accumulations of acetate, Asn, Tyr, Ser, Gly, Sar and glycerol, and the consumptions of lactate and lipids in intact zebrafish over the storage time. The changes of metabolites during fish spoilage were due to enzymatic autolysis at initial stage and microorganism activities at higher rates afterward (Ciampa, Picone, Laghi, Nikzad, & Capozzi, 2012; Nair, Joshi, Boricha, Haldar, & Chatterjee, 2016). The spoilage bacteria rapidly increased during the early stage of fish spoilage, generating a lot of proteolytic and hydrolytic enzymes (Ghaly et al., 2010). In our results, although several metabolites in uninoculated samples changed during 10 d storage due to autolytic enzymes, more changed metabolites and higher levels were observed in inoculated samples, which were caused by the activity of *S. baltica*.

3.4.1. Changes of amino acids and peptides

Amino acids and peptides were main metabolites identified in two fish models. Most of quantified amino acids were observed with significantly rising and declining trends in fish sticks and broths during spoilage, respectively.

Odagami, Morita, Takama, and Suzuki (1994) reported that trypsin-like enzymes with relatively broad spectrum towards substrates were produced by *S. putrefaciens*. Izuchukwu (2017) revealed different protease groups secreted by *S. baltica* strains, including aspartic, serine, metallo and cysteine proteases. Li et al. (2020) analysed complete genome sequencing of *S. baltica* 128 and reported that there are 8.66% genes were related to amino acid transport and metabolism. They also found serine protease in this strain, which is the major extracellular proteases in *Shewanella* (Kulakova, Galkin, Kurihara, Yoshimura, & Esaki, 1999). In addition, Leyva-Díaz, Poyatos, Barghini, Gorrasi, and Fenice (2017) suggested that the proteolytic pathways of *S. baltica* KB30 was probably more favoured than lipolytic and glycolytic pathways according to respirometry results. Therefore, the release of free amino acids and anserine in fish sticks were mainly derived from the hydrolysis of proteins, which was accelerated by the extracellular proteases from *S. baltica*. The highest increases of amino acids were observed in strain ABa4, showing that strain ABa4 might produce more proteases than strain ABe2 and BBe1. However, the contents of Gly and creatine decreased during storage, probably because the accumulation rate of proteolysis and transformation from other substances were lower than the degradation rates.

On the other hand, during the preparation of fish broths, proteins and other macromolecules were eliminated. Moreover, the fish broths were treated with heating, which inactivated autolytic enzymes in fish tissues. Hence, protein hydrolysis might not occur continuously in inoculated fish broths during spoilage. It has been reported that amino acids can be utilised by spoilage bacteria as nitrogen and carbon sources for heterotrophic metabolism, and further metabolised into volatile compounds (Leyva-Díaz et al., 2017; Zhu, Wu, et al., 2015). The results of fish broths revealed that *S. baltica* was able to decompose amino acids to other metabolites.

3.4.2. Changes of biogenic amines

Biogenic amines are regarded as quality indices of seafood (Ghaly et al., 2010). In our study, TMA, DMA, TMAO, putrescine and tyramine were identified, and three of them were quantified. The reduction of TMAO and production of TMA occurred during fish storage due to the activities of TMAO demethylase in fish tissue and spoilage bacteria (Debevere et al., 2001; Ghaly et al., 2010). These reactions provide energy to bacterial respiration, releasing off-flavour of fish (Odeyemi, Burke, Bolch, & Stanley, 2018). In *S. baltica*, TMAO reductase and amino acid decarboxylase were found, which catalyse the formations of TMA and amino acid-derived amines, respectively (Zhao, Zhu, Ye, Ge, & Li, 2016). Besides, TMA is associated with the breakdown of choline and betaine during spoilage (Zhao, Wu, et al., 2019). In the present study, the increased DMA (2-fold) and tyramine (0.5-fold) in fish sticks and TMA (2.5-fold) in fish broths were formed by *S. baltica*. There was no significant difference between TMA content in the control and in inoculated fish sticks on day 10, which could be explained by that TMA was further degraded to DMA during storage (Castejón, García-Segura, Herrera, & Cambero, 2016). In fish broths, strain BBe1 showed highest levels of TMA and tyramine, suggesting that it could form more biogenic amines.

3.4.3. Changes of nucleotides and their derivatives

Nucleotides are of importance to fish and seafood, because they can be considered as both taste compounds and quality indices. As indicated by Zhao, Wu, et al. (2019), there is a distinct metabolic pathway among nucleotides and derivatives, which includes a series of sequential degradations (from ATP, ADP, AMP, adenosine, IMP to inosine and hypoxanthine). IMP can provide umami and sweet flavour, while inosine and hypoxanthine contribute to bitterness in meat (Lou et al., 2018). ATP,

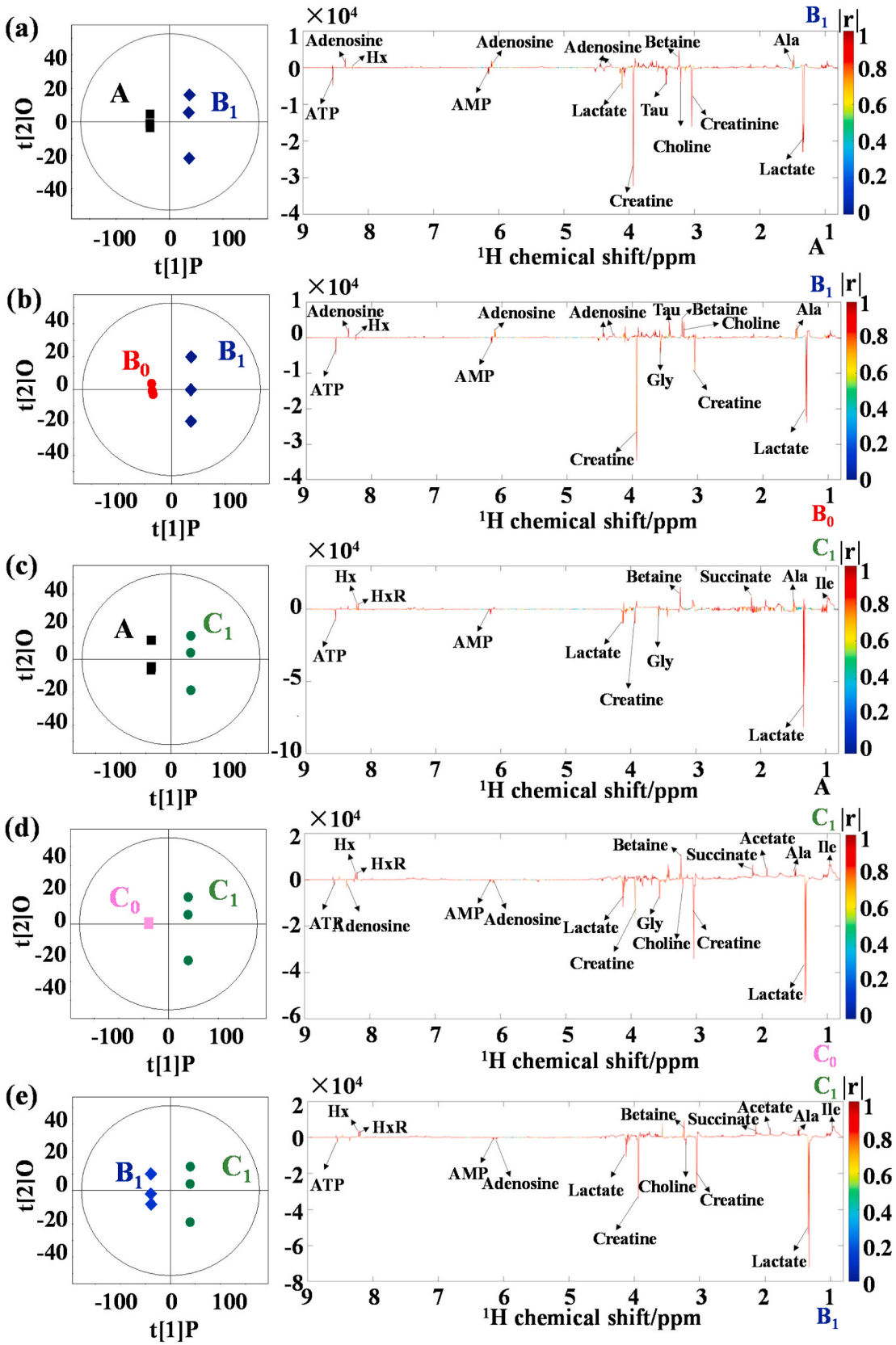

Fig. 3. OPLS-DA cross-validated score plots (left) and coefficient-coded loading plots (right) of pairwise groups for fish sticks inoculated with strain ABa4. (a): Comparison results between day 0 (A) and 4 (B_1); (b): Comparison results between without (B_0) and with (B_1) strain on day 4; (c): Comparison results between day 0 (A) and 10 (C_1); (d): Comparison results between without (C_0) and with (C_1) strain on day 10; (e): Comparison results between day 4 (B_1) and 10 (C_1).

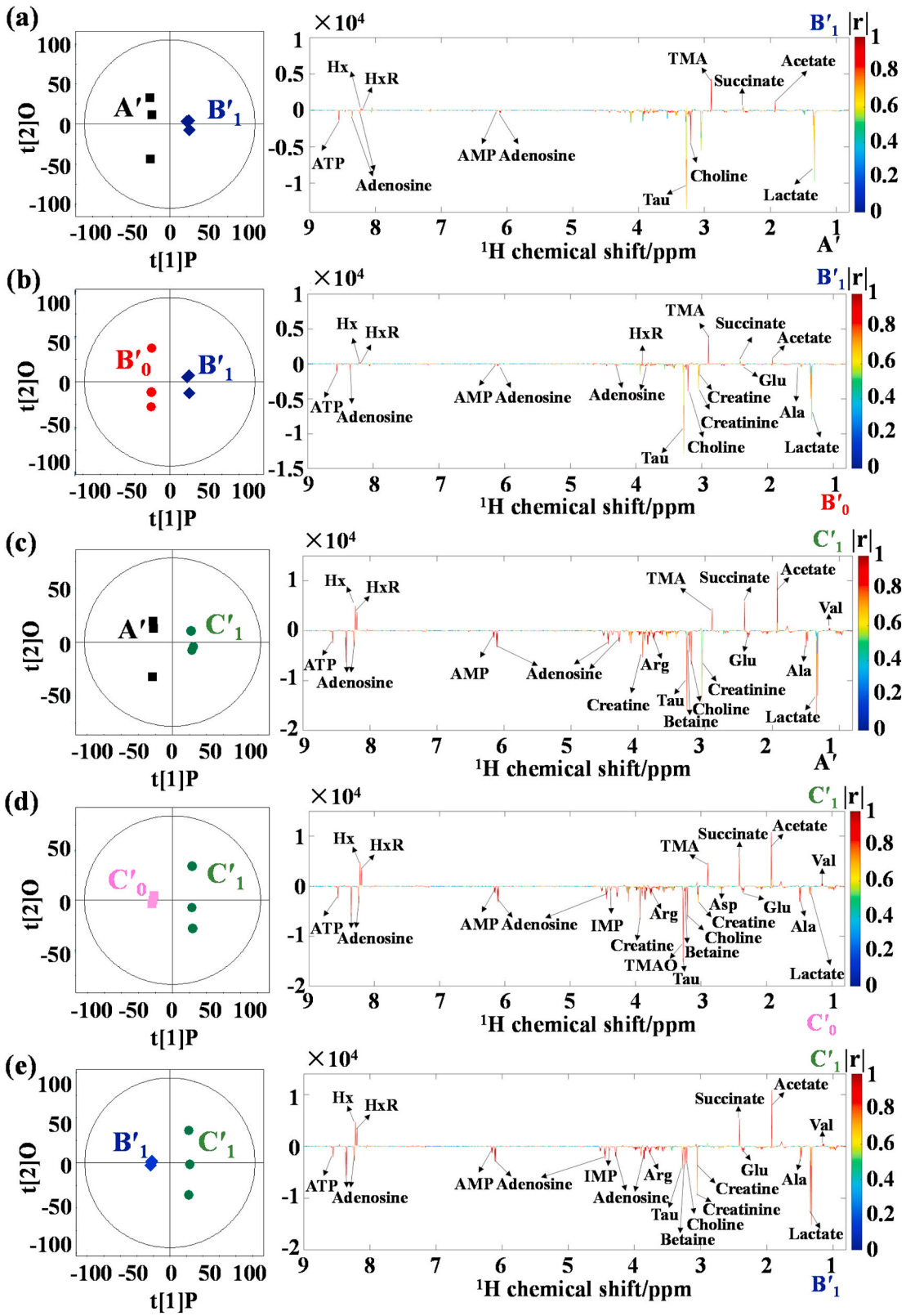

Fig. 4. OPLS-DA cross-validated score plots (left) and coefficient-coded loading plots (right) of pairwise groups for fish broths inoculated with strain ABa4. (a): Comparison results between day 0 (A′) and 4 (B'_1); (b): Comparison results between without (B'_0) and with (B'_1) strain on day 4; (c): Comparison results between day 0 (A′) and 10 (C'_1); (d): Comparison results between without (C'_0) and with (C'_1) strain on day 10; (e): Comparison results between day 4 (B'_1) and 10 (C'_1).

Table 1
Metabolite contents in fish sticks inoculated with *Shewanella baltica* during storage.

Metabolite	Mean (mg/g)								
	A	B_0	B_1	B_2	B_3	C_0	C_1	C_2	C_3
Leu	0.174^{d}	0.164^{e}	0.188^{c}	0.163^{e}	0.161^{e}	0.162^{e}	0.464^{a}	0.456^{b}	0.454^{b}
Ile	0.253^{c}	0.244^{cd}	0.250^{c}	0.224^{de}	0.235^{cde}	0.219^{e}	0.433^{a}	0.389^{b}	0.391^{b}
Val	0.053^{b}	0.043^{c}	0.035^{c}	0.029^{e}	0.039^{cd}	0.025^{e}	0.064^{a}	0.055^{b}	0.056^{b}
Met	0.076^{e}	0.090^{d}	0.100^{c}	0.093^{cd}	0.078^{e}	0.097^{cd}	0.260^{a}	0.250^{b}	0.256^{ab}
Lys	0.377^{c}	0.339^{d}	0.326^{de}	0.310^{e}	0.339^{d}	0.292^{f}	0.525^{a}	0.502^{b}	0.498^{b}
Phe	0.116^{d}	0.113^{de}	0.118^{d}	0.100^{f}	0.106^{ef}	0.111^{e}	0.303^{a}	0.296^{b}	0.290^{c}
Ala	0.505^{cd}	0.487^{e}	0.511^{c}	0.434^{g}	0.497^{d}	0.472^{f}	0.644^{a}	0.625^{b}	0.631^{b}
Tyr	0.136^{e}	0.143^{de}	0.159^{c}	0.135^{e}	0.149^{d}	0.163^{c}	0.251^{a}	0.240^{b}	0.239^{b}
Gly	1.583^{b}	1.612^{a}	1.378^{d}	1.362^{d}	1.501^{c}	1.574^{b}	1.277^{e}	1.231^{f}	1.239^{f}
Tau	2.311^{a}	2.002^{e}	2.235^{b}	2.184^{c}	2.131^{d}	1.994^{e}	2.242^{b}	2.175^{c}	2.172^{c}
Gln	0.339^{c}	0.307^{de}	0.319^{d}	0.293^{ef}	0.315^{d}	0.283^{f}	0.953^{a}	0.927^{b}	0.922^{b}
Glu	0.322^{c}	0.303^{cd}	0.312^{c}	0.273^{d}	0.291^{cd}	0.278^{d}	0.701^{a}	0.660^{b}	0.645^{b}
Asp	0.238^{c}	0.239^{c}	0.309^{b}	0.248^{c}	0.244^{c}	0.246^{c}	0.549^{a}	0.523^{a}	0.525^{a}
Arg	0.150^{cd}	0.142^{d}	0.168^{c}	0.168^{c}	0.155^{cd}	0.202^{b}	0.470^{a}	0.461^{a}	0.454^{a}
Anserine	0.080^{b}	0.063^{c}	0.064^{c}	0.066^{c}	0.065^{c}	0.061^{c}	0.127^{a}	0.120^{a}	0.132^{a}
Lactate	7.022^{a}	6.549^{b}	6.392^{c}	6.311^{d}	6.033^{e}	5.576^{f}	3.011^{g}	2.900^{h}	2.908^{h}
Acetate	0.014^{d}	0.014^{d}	0.017^{c}	0.013^{d}	0.015^{d}	0.014^{d}	0.111^{a}	0.106^{b}	0.106^{b}
Succinate	0.014^{e}	0.019^{d}	0.024^{c}	0.021^{d}	0.025^{c}	0.021^{d}	0.044^{a}	0.041^{b}	0.041^{b}
Creatine	7.925^{a}	7.416^{b}	6.885^{c}	6.933^{c}	6.885^{c}	6.334^{d}	6.236^{e}	6.083^{f}	6.095^{f}
Glucose	0.293^{a}	0.261^{b}	0.244^{c}	0.253^{bc}	0.252^{bc}	0.224^{d}	0.161^{e}	0.153^{e}	0.160^{e}
2,3-butanediol	0.059^{a}	0.048^{b}	0.045^{b}	0.042^{bc}	0.046^{b}	0.037^{c}	0.061^{a}	0.058^{a}	0.060^{a}
TMA	0.010^{a}	0.009^{b}	0.008^{bc}	0.008^{bc}	0.008^{bc}	0.007^{c}	0.008^{bc}	0.008^{bc}	0.008^{bc}
DMA	0.003^{c}	0.002^{d}	0.004^{c}	0.003^{c}	0.003^{c}	0.002^{d}	0.007^{a}	0.005^{b}	0.005^{b}
Tyramine	0.066^{c}	0.070^{bc}	0.071^{b}	0.065^{c}	0.071^{b}	0.067^{b}	0.104^{a}	0.103^{a}	0.103^{a}
ATP	0.195^{a}	0.172^{b}	0.151^{bc}	0.166^{bc}	0.152^{bc}	0.143^{c}	0.104^{d}	0.106^{d}	0.120^{d}
AMP	2.321^{a}	2.065^{b}	1.238^{e}	1.390^{c}	1.319^{d}	0.816^{f}	0.795^{f}	0.796^{f}	0.798^{f}
Adenosine	1.175^{d}	1.212^{c}	1.347^{b}	1.324^{b}	1.341^{b}	1.556^{a}	0.887^{e}	0.860^{f}	0.849^{f}
IMP	1.741^{a}	1.673^{b}	1.001^{d}	1.263^{c}	0.978^{e}	0.676^{f}	0.351^{g}	0.344^{g}	0.334^{g}
Inosine	0.095^{f}	0.111^{f}	0.151^{e}	0.178^{de}	0.168^{de}	0.237^{c}	0.833^{a}	0.815^{ab}	0.797^{b}
Hypoxanthine	0.041^{f}	0.050^{e}	0.081^{d}	0.089^{d}	0.086^{d}	0.113^{c}	0.407^{a}	0.388^{b}	0.386^{b}
Betaine	0.114^{g}	0.123^{f}	0.171^{c}	0.169^{c}	0.159^{d}	0.135^{e}	0.212^{a}	0.207^{b}	0.210^{ab}
Choline	0.083^{ab}	0.078^{d}	0.079^{cd}	0.083^{ab}	0.081^{bc}	0.084^{a}	0.060^{e}	0.058^{ef}	0.057^{f}
Creatinine	0.218^{bc}	0.180^{de}	0.212^{c}	0.178^{de}	0.171^{e}	0.186^{d}	0.237^{a}	0.218^{bc}	0.227^{ab}

A: fish stick on day 0; B_0: fish stick without strain on day 4; B_1: fish stick with strain ABa4 on day 4; B_2: fish stick with strain ABe2 on day 4; B_3: fish stick with strain BBe1 on day 4; C_0: fish stick without strain on day 10; C_1: fish stick with strain ABa4 on day 10; C_2: fish stick with strain ABe2 on day 10; C_3: fish stick with strain BBe1 on day 10.
a-h.Different letters mean significant difference among different samples ($P < 0.05$, along the lines).

ADP, AMP, IMP, inosine and hypoxanthine are the constituents of K value, which is closely correlated with the freshness of fish (Vazquez-Ortiz, Pacheco-Aguilar, Lugo-Sanchez, & Villegas-Ozuna, 1997).

Sterile fish models had relatively high amount of AMP (2.321 mg/g in sticks, 0.712 mg/mL in broths) and IMP (1.741 mg/g in sticks, 0.435 mg/mL in broths). Grigorakis, Taylor, and Alexis (2003) observed the breakdown of ATP during the spoilage of gilthead sea bream. Li et al. (2020) detected the gene of nucleotidase in *S. baltica*. In our study, nucleotides were degraded in uninoculated fish sticks during spoilage but presented no change in uninoculated broths, suggesting that endogenous nucleotidase in fish tissue was still activated to impair fish freshness at chilled storage. In both inoculated fish sticks and broths, the concentrations of ATP, AMP, adenosine and IMP significantly decreased, accompanied with the increases of inosine and hypoxanthine, compared to the controls. These results indicated that the degradation reactions occurred in fish models by nucleotidase from *S. baltica*. Strain BBe1 showed highest accumulations of inosine and hypoxanthine on day 4 and reductions of nucleotides on day 10, but showed lowest inosine and hypoxanthine levels on day 10. It might be due to that more inosine and hypoxanthine were further degraded by strain BBe1 at later stage of spoilage. Our findings were in accordance with Liu et al. (2018), who reported that fast degradations of nucleotides were found in bighead carp inoculated with *S. putrefaciens*.

3.4.4. Changes of sugars and organic acids

Carbohydrates and carboxylic acids are favourite carbon sources for bacterial growth. Glucose and lactate are usually consumed by microorganisms at first instance in the spoilage of fish (Dainty, 1996). It has been reported that *S. baltica* was able to degrade sucrose, gluconic acid, D-glucose, dextrin, maltose and lactic acid, implying the presence of correlative catalytic enzymes in *S. baltica* (Deng et al., 2014). Lactate was reported to be the H-donor for the reduction of TMAO in fish tissue during spoilage, and acetate and TMA were suggested as the end products (Debevere et al., 2001). Moreover, succinate and acetate can be transformed from corresponding precursors by *Shewanella* (McLean et al., 2008). Ciampa et al. (2012) showed that glucose and lactate decreased by 40% for Bogue fish storage at 4 °C, whilst succinate and acetate levels elevated. Such findings are in line with our data, which demonstrate that *S. baltica* utilised sugars and lactate for metabolism, and produced succinate and acetate in fish models during refrigerated storage.

3.5. Changes of TVB-N and TVC of fish models inoculated with S. baltica during spoilage

To validate the NMR results, TVB-N and TVC of fish models during spoilage are shown in Fig. 5. In uninoculated samples during storage, TVB-N of sticks slightly increased but there was no change of broths, which further confirmed the autolytic proteases in sterile fish sticks and inactivated enzymes in sterile fish broths. It can be readily seen that TVB-N increased dramatically in both inoculated fish sticks and broths during spoilage, which was related to the formation of volatile nitrogen-containing compounds by *S. baltica*. Similar results about inoculated fish sticks and broths have been reported by Liu et al. (2018) and Gu et al. (2013), respectively. These results verified that *S. baltica* was a kind of spoilage bacterium of fish in terms of its ability of degradation of amino

Table 2
Metabolite contents in fish broths inoculated with *Shewanella baltica* during storage.

Metabolite	Mean (mg/mL)								
	A′	B′$_0$	B′$_1$	B′$_2$	B′$_3$	C′$_0$	C′$_1$	C′$_2$	C′$_3$
Leu	0.153^{ab}	0.156^{a}	0.151^{b}	0.149^{b}	0.149^{b}	0.148^{b}	0.130^{d}	0.132^{d}	0.142^{c}
Ile	0.151^{a}	0.151^{a}	0.148^{ab}	0.145^{bc}	0.147^{abc}	0.148^{ab}	0.129^{e}	0.135^{d}	0.142^{c}
Val	0.023^{d}	0.023^{d}	0.023^{d}	0.023^{d}	0.024^{d}	0.022^{d}	0.047^{c}	0.054^{a}	0.051^{b}
Met	0.056^{a}	0.053^{ab}	0.051^{c}	0.053^{ab}	0.054^{ab}	0.052^{bc}	0.048^{d}	0.050^{cd}	0.052^{bc}
Lys	0.285^{a}	0.287^{a}	0.282^{a}	0.286^{a}	0.286^{a}	0.282^{a}	0.270^{a}	0.271^{a}	0.278^{a}
Phe	0.121^{ab}	0.126^{a}	0.123^{a}	0.118^{ab}	0.122^{ab}	0.120^{ab}	0.123^{ab}	0.116^{b}	0.125^{a}
Ala	0.351^{a}	0.351^{a}	0.338^{b}	0.340^{b}	0.342^{ab}	0.339^{b}	0.273^{d}	0.294^{c}	0.344^{ab}
Tyr	0.118^{a}	0.117^{a}	0.107^{c}	0.109^{bc}	0.115^{ab}	0.111^{abc}	0.107^{c}	0.109^{bc}	0.105^{c}
Gly	0.446^{a}	0.446^{a}	0.438^{a}	0.432^{a}	0.438^{a}	0.441^{a}	0.427^{a}	0.448^{a}	0.442^{a}
Tau	1.551^{a}	1.553^{a}	1.503^{ab}	1.473^{b}	1.485^{b}	1.511^{ab}	1.470^{b}	1.503^{ab}	1.505^{ab}
Gln	0.340^{ab}	0.344^{a}	0.329^{bc}	0.338^{ab}	0.339^{ab}	0.332^{abc}	0.312^{d}	0.311^{d}	0.324^{cd}
Glu	0.309^{a}	0.299^{ab}	0.291^{bc}	0.287^{bc}	0.292^{bc}	0.290^{bc}	0.227^{d}	0.240^{d}	0.278^{c}
Asp	0.195^{ab}	0.198^{a}	0.187^{b}	0.195^{ab}	0.196^{ab}	0.194^{ab}	0.133^{c}	0.131^{c}	0.129^{c}
Arg	0.281^{a}	0.289^{a}	0.265^{b}	0.278^{a}	0.285^{a}	0.277^{ab}	0.176^{e}	0.204^{d}	0.235^{c}
Anserine	0.027^{a}	0.022^{b}	0.024^{ab}	0.024^{ab}	0.023^{b}	0.025^{ab}	0.017^{c}	0.017^{c}	0.015^{c}
Lactate	3.089^{a}	3.102^{a}	2.963^{b}	2.932^{b}	2.949^{b}	2.961^{b}	2.716^{c}	2.777^{c}	2.913^{b}
Acetate	0.022^{f}	0.024^{f}	0.041^{e}	0.039^{e}	0.049^{d}	0.042^{e}	0.122^{c}	0.133^{b}	0.146^{a}
Succinate	0.008^{f}	0.009^{f}	0.016^{e}	0.016^{e}	0.024^{d}	0.009^{f}	0.078^{c}	0.088^{b}	0.125^{a}
Creatine	1.517^{a}	1.513^{a}	1.464^{b}	1.442^{bc}	1.429^{bc}	1.464^{b}	1.422^{c}	1.444^{bc}	1.424^{c}
Glucose	0.103^{a}	0.102^{a}	0.065^{c}	0.069^{c}	0.095^{b}	0.100^{ab}	0.063^{c}	0.046^{e}	0.055^{d}
Maltose	0.015^{a}	0.010^{b}	0.011^{ab}	0.011^{ab}	0.012^{ab}	0.011^{b}	0.012^{ab}	0.012^{ab}	0.011^{ab}
2,3-butanediol	0.034^{a}	0.03^{b}	0.031^{b}	0.029^{b}	0.032^{ab}	0.030^{b}	0.032^{ab}	0.032^{ab}	0.030^{b}
TMA	0.007^{d}	0.007^{d}	0.019^{b}	0.010^{cd}	0.012^{c}	0.007^{d}	0.022^{b}	0.024^{ab}	0.027^{a}
DMA	0.011^{bc}	0.011^{bc}	0.011^{bc}	0.013^{a}	0.012^{ab}	0.011^{bc}	0.010^{c}	0.010^{c}	0.010^{c}
Tyramine	0.029^{ab}	0.030^{a}	0.028^{ab}	0.029^{ab}	0.029^{ab}	0.029^{ab}	0.026^{c}	0.026^{c}	0.028^{b}
ATP	0.067^{ab}	0.067^{ab}	0.065^{ab}	0.063^{ab}	0.067^{ab}	0.068^{a}	0.012^{c}	0.005^{d}	0.001^{d}
AMP	0.712^{a}	0.712^{a}	0.681^{a}	0.690^{a}	0.683^{a}	0.681^{a}	0.251^{b}	0.226^{b}	0.270^{b}
Adenosine	0.712^{a}	0.718^{a}	0.643^{c}	0.658^{c}	0.593^{d}	0.696^{b}	0.010^{e}	0.005^{e}	0.006^{e}
IMP	0.435^{a}	0.432^{a}	0.403^{b}	0.411^{b}	0.406^{b}	0.423^{a}	0.023^{c}	0.004^{d}	0.003^{d}
Inosine	0.126^{cd}	0.117^{d}	0.150^{cd}	0.143^{cd}	0.181^{c}	0.132^{cd}	0.715^{a}	0.713^{a}	0.610^{b}
Hypoxanthine	0.062^{c}	0.062^{c}	0.079^{c}	0.081^{c}	0.091^{c}	0.073^{c}	0.367^{a}	0.364^{a}	0.323^{b}
Betaine	0.070^{ab}	0.073^{a}	0.068^{b}	0.070^{ab}	0.070^{ab}	0.070^{ab}	0.044^{c}	0.046^{c}	0.067^{b}
Choline	0.054^{a}	0.053^{ab}	0.040^{c}	0.040^{c}	0.052^{ab}	0.052^{ab}	0.022^{d}	0.021^{d}	0.051^{b}
Creatinine	1.780^{a}	1.771^{ab}	1.726^{bc}	1.742^{abc}	1.724^{bc}	1.724^{bc}	1.717^{bc}	1.732^{abc}	1.708^{c}

A′: fish broth on day 0; B′$_0$: fish broth without strain on day 4; B′$_1$: fish broth with strain ABa4 on day 4; B′$_2$: fish broth with strain ABe2 on day 4; B′$_3$: fish broth with strain BBe1 on day 4; C′$_0$: fish broth without strain on day 10; C′$_1$: fish broth with strain ABa4 on day 10; C′$_2$: fish broth with strain ABe2 on day 10; C′$_3$: fish broth with strain BBe1 on day 10.
[a-g].Different letters mean significant difference among different samples ($P < 0.05$, along the lines).

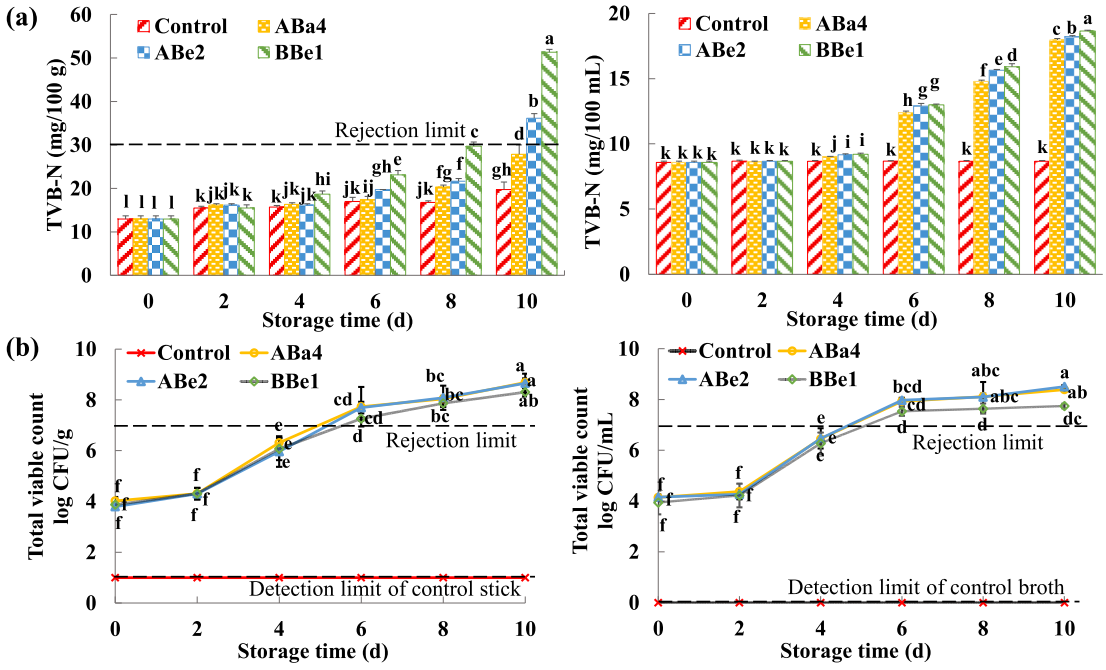

Fig. 5. Changes in TVB-N (a) and TVC (b) of fish sticks (left) and broths (right) inoculated with *S. baltica* during storage. Different letters indicate a significant difference ($P < 0.05$). The TVC detection limits of inoculated fish sticks and broths were 2 log CFU/g and 1 log CFU/mL, respectively.

acids and proteins.

Fig. 5b shows the growth of different *S. baltica* strains in fish sticks and broths during refrigerated storage. All strains presented specific growth curves in two fish models, which were quite similar with an earlier observation by Zhao et al. (2016). Interestingly, strain BBe1 reached a relatively lower population than other strains during storage, but showed higher TVB-N and capability to produce biogenic amines and to degrade nucleotides, implying that strain BBe1 might have higher spoilage potential than strain ABa4 and ABe2.

3.6. Schematic illustration

According to the results of multivariate data analysis and absolute quantification, an assumptive schematic of *S. baltica* activity on metabolic changes of fish sticks and broths was proposed (Fig. 6). There are four main metabolic pathways in this study: peptide and amino acid, nitrogen, nucleotide, and carbohydrate pathways. Proteins were hydrolysed, releasing amino acids and peptides in fish sticks, while amino acids and peptides were decomposed in fish broths. Nitrogen, nucleotide and carbohydrate metabolisms of *S. baltica* in fish sticks and broths are similar. Amino acids, TMAO and other nitrogen-containing compounds were transformed to biogenic amines, including TMA, DMA, tyramine etc (Castejón et al., 2016). Nucleotides underwent gradual degradations to form inosine and hypoxanthine by nucleotidase of *S. baltica* (Odeyemi et al., 2018). Sugars and lactate were consumed by *S. baltica*, producing acetate and succinate (Ciampa et al., 2012; Zhao, Wu, et al., 2019). In addition, different metabolisms can be interlinked by some transformations, such as the reaction from lactate to TMA.

4. Conclusion

In conclusion, NMR spectroscopy and multivariate data analysis provided comprehensive information of the metabolic changes in fish sticks and broths inoculated with different *S. baltica* strains during spoilage. The activities of *S. baltica* involved in four main metabolic pathways, including amino acid and peptide, nitrogen, nucleotide and carbohydrate pathways. *S. baltica* increased the levels of free amino acids in fish sticks but decreased their concentrations in fish broths. In both inoculated fish sticks and broths, amino acids and TMAO were transformed to biogenic amines; adenine nucleotides were gradually degraded to form inosine and hypoxanthine; sugars and lactate were consumed and other organic acids were produced. In addition, the increased TVB-N and TVC further validated NMR results. These findings show that NMR spectroscopy was effective to study the metabolism of *S. baltica* in fish models.

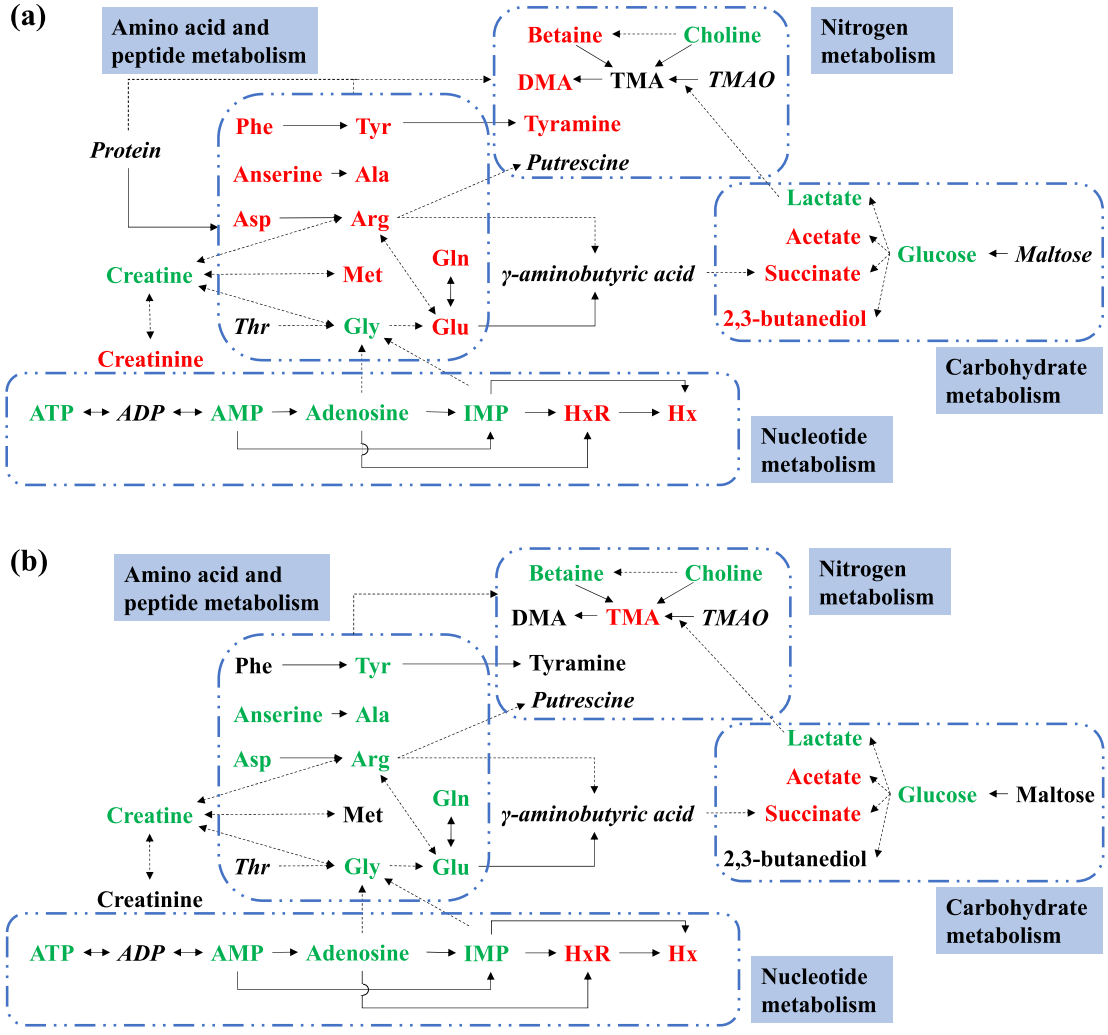

Fig. 6. Proposed schematic on the metabolic changes in fish sticks (a) and broths (b) inoculated with *S. baltica* during storage. Metabolite in an italic font is not quantified or identified; metabolite coloured in red, green or black represents a higher, lower or similar level as compared to the control, respectively; transformation with dotted or solid line means that this reaction has or does not have intermediate product(s). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

CRediT authorship contribution statement

Xiaowei Lou: Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing - original draft. **Doudou Zhai:** Data curation, Formal analysis, Investigation. **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.

Acknowledgements

This study was financially supported by the Singapore Ministry of Education Academic Research Fund Tier 1 (R-160-000-A40-114), Natural Science Foundation of Jiangsu Province (BK20181184), and an industry project supported by Fujian Changle Juquan Food Co., Ltd (R-160-000-A73-597).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2020.107697.

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