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The roles of bacteria in the biochemical changes of chill-stored bighead carp (*Aristichthys nobilis*): Proteins degradation, biogenic amines accumulation, volatiles production, and nucleotides catabolism

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ABSTRACT

This study investigated the biochemical changes (proteins degradation, total volatile basic nitrogen, biogenic amines, volatile organic compounds, nucleotides catabolism and related enzymes) of bighead carp samples inoculated with four different bacteria (*Shewanella putrefaciens*, *Aeromonas sobria*, *Acinetobacter bohemicus*, and *Pseudomonas helmanticensis*) during storage at 4 ± 1 °C. *A. sobria* exhibited the strongest proteolytic activity. *A. sobria*, *P. helmanticensis*, and *S. putrefaciens* were responsible for putrescine production, whereas *S. putrefaciens* was the sole producer of cadaverine. Alcohols and S-compounds were mainly released by *A. sobria* and *S. putrefaciens*, respectively. The fastest degradation rates of hypoxanthine riboside and hypoxanthine were found in samples inoculated with *P. helmanticensis* and *S. putrefaciens*. Inosine nucleosidase was mainly resulted by *A. sobria*, *P. helmanticensis* and *S. putrefaciens*, whereas xanthine oxidase was derived from both fish muscle and secretions of *P. helmanticensis* and *S. putrefaciens*.

1. Introduction

Fish spoilage is mainly caused by a group of bacteria, which is called specific spoilage organisms (SSOs). These bacteria are originated from the initial microbiota and become dominant at the onset of spoilage (Gram & Huss, 1996). Previous studies have showed that SSOs are associated with undesirable biochemical changes in fish, such as proteins degradation, nucleotides catabolism, biogenic amines (BAs) accumulation and volatile organic compounds (VOCs) production, among others (Gram & Huss, 1996; Huis in't Veld, 1996; Sofos, et al., 2013). Putrescine and cadaverine are two principal BAs in freshwater fish which are produced from bacterial decarboxylation of specific amino acids (Shi, Cui, Lu, Shen, & Luo, 2012; Zhang, Qin, Luo, & Shen, 2014). Lots of VOCs, commonly include alcohols, aldehydes, ketones, sulfur compounds and other molecules, are reported to be bacterial metabolites (Casaburi, Piombino, Nychas, Vilani, & Ercolini, 2015). Protein constitutes approximately 15–20% of fish muscle, of which the degradation may influence muscle texture, flavor, taste, and functional properties (Aro, et al., 2010; Halim, Yusof, & Sarbon, 2016). Bacterial actions are important contributors to the degradation of fish proteins (Ge, Xu, Xia, Jiang, & Jiang, 2016; Sofos et al., 2013). Venugopal, Alur, and Lewis (1983) determined the *in vitro* proteolytic activity of an extracellular protease isolated from *Pseudomonas marinoglutinosa* [a spoilage bacterium from Indian mackerel (*Rastrelliger kanagurta*)], and found this enzyme showed high activity against myofibrillar proteins. Nucleotides in fish muscle are mainly derived from catabolism of adenosine triphosphate (ATP), which follow the degradative sequence of ATP → adenosine diphosphate (ADP) → adenosine monophosphate (AMP) → inosine 5′-monophosphate (IMP) → hypoxanthine riboside (HxR) → hypoxanthine (Hx) → xanthine (Xa) → uric acid (UA) (Hong, Regenstein, & Luo, 2017). IMP and Hx, which are two intermediate nucleotides, act as a flavor enhancer and a bitter compound, respectively (Hong et al., 2017). Shiba, Shiraki, Furushita, and Maeda (2014) and Li et al. (2017) investigated the catabolism of nucleotides in non-sterile and sterile fish fillets, reporting that autolysis may lead to the conversion of ATP to IMP, while bacteria may play prominent roles in the hydrolysis of HxR. Hitherto, the contributions of SSOs to the production of VOCs and BAs have been studied in freshwater fish, marine fish and its related products (Wang et al., 2017; Jørgensen, Huss, & Dalgaard, 2000; Stohr, Joffraud, & Leroi, 2001; Joffraud et al., 2006; Macé et al., 2013). However, limited studies have been found on the contributions of bacterial species to proteins degradation and nucleotides catabolism. Given the importance of these two kinds of biochemical changes, it is worthwhile to study the roles of bacterial species

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in proteins degradation and nucleotides catabolism.

The spoilage microbiota of temperate fish stored aerobically are generally dominated by psychrotrophic Gram-negative bacteria, such as *Aeromonas*, *Pseudomonas*, and *Shewanella* (Parlapani, Meziti, Kormas, & Boziaris, 2013; Wang, Luo, Huang, & Xu, 2014; Zhang, Li, Li, Liu, & Luo, 2015; Huang, Liu, Jia, & Luo, 2017). Bighead carp (*Aristichthys nobilis*) is one of the main freshwater fish species from temperate waters. In our previous studies, 15 different species belonging to the genera of *Aeromonas*, *Pseudomonas*, *Shewanella*, and *Acinetobacter* were isolated from spoiled bighead carp. In this study, the roles of the top four predominant species (*Shewanella putrefaciens*, *Aeromonas sobria*, *Acinetobacter bohemicus*, and *Pseudomonas helmanticensis*) in proteins degradation, nucleotides catabolism, biogenic amines accumulation and volatiles production were explored in fish matrix. This work may lead to a comprehensive and deep insight into the mechanism underlying biochemical changes of freshwater fish during chilled storage.

2. Material and methods

2.1. Bacteria strains

All strains were previously isolated from spoiled bighead carp fillets which were stored aerobically at 4 ± 1 °C for 8 days. Twenty-five grams of spoiled flesh were homogenized with 0.9% sterile saline solution and 10-fold serially diluted. Microbiological number was determined by cultivation on plate count agar (Beijing Land Bridge Technology, Co. LTD, China) at 30 °C for 72 h. All colonies were isolated, purified, submitted to DNA extraction, and identified by 16S rRNA gene sequencing of about 1400 bp with primer 27f (5′-GAGATT TGATCCTGGCTCAG-3′) and 1495r (5′-CTACGGCTACCTTGTTA CGA-3′).

A total of 115 isolates were obtained, and belonged to 4 genera: *Shewanella* (47/115), *Aeromonas* (38/115), *Acinetobacte* (12/115), and *Pseudomonas* (18/115). *Shewanella putrefaciens* (47/115), *Aeromonas sobria* (37/115), *Acinetobacter bohemicus* (8/115), and *Pseudomonas helmanticensis* (6/115) were identified as the top four predominant species. Other species comprised 1–3 strains. In this study, the top four dominant species were used, and each group was represented by 5 isolates of the same species.

2.2. Preparation of sterile muscle blocks

Thirty bighead carp (length: 52.70 ± 1.86 cm, weight: 1608 ± 69 g) were purchased from an aquatic product market in Beijing, China in April 2017.The bighead carp were then transported to the laboratory alive in polyethylene bags with water and oxygen. The fish were stunned, scaled, gutted, headed and washed, after which their outer surface were wiped with 75% ethanol solution. The backbone muscle (with skin) were excised using sterile cutting boards and knives, and placed in sterile bags. Fillets were then skinned and cut into blocks (about 3 cm × 3 cm × 2 cm) in a clean bench. Twelve blocks were obtained from each fish. Blocks were then immersed in 0.5% (v/v) formalin solution for 20 s and rinsed with sterile water for three times. The sterile blocks were stored at 4 ± 1 °C until required.

2.3. Strain cultivation, sample inoculation and storage

Strains were pre-cultured in tryptic soy broth at 30 °C on a gyratory shaker (100 rpm/min) until the microbiological number reached 9 log CFU/g. Mixtures of 5 isolates belonging to the same species were pooled and serially diluted (1:10, 0.9% NaCl solution) to obtain a microbiological number of 6 log CFU/g.

The sterile blocks were divided randomly into 5 groups: one control group and four inoculated groups. For each inoculated group, every 24 blocks were immersed in 500 mL inoculation mixture for 10 min to achieve an inoculated level of 4–5 log CFU/g. For the control group, blocks were immersed in sterile NaCl solution. After inoculation, every 3 blocks were packed in a sterile polyvinyl chloride bag and stored at 4 ± 1 °C. Three packages in each group were randomly selected and used for analyses every 3 days.

2.4. Microbiological numbers

Samples were homogenized with 10-fold sterile NaCl solution for 30 s in a stomacher, and then serially diluted (1:10, NaCl solution). Samples (0.1 mL) of each dilution were spread on plate count agar, and incubated at 30 ± 1 °C for 72 ± 2 h. Microbiological number was expressed as log CFU/g.

2.5. Proteins preparation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Two grams of minced muscle were homogenized with 15 mL cold deionized water for 1 min, and the mixture was centrifuged at 10 000*g* for 10 min. The supernatant was collected, and the pellet was homogenized with 15 mL cold NaCl solution (0.3%) and centrifuged at 10 000*g* for 10 min. The combined supernatant was used for the analysis of sarcoplasmic proteins. The pellet was homogenized with 30 mL 0.6 mol/L NaCl-20 mM Tris-maleate buffer (pH 7.0). Then the mixture was placed at 4 °C for 1 h, and centrifuged at 10 000*g* for 10 min. Eight milliliters supernatant mixed with 30 mL cold deionized water was vortexed for 1 min. The mixture was centrifuged, and the pellet was dissolved in 8 mL 0.6 mol/L NaCl solution to obtain myofibrillar proteins. Protein concentration was measured by the biuret method, and was adjusted to 2 mg/mL. Protein samples were mixed with loading buffer (0.1 mol/L Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, and 0.1% bromophenol blue) at a ratio of 1:1 (v/v), and the mixtures were heated in boiled water for 5 min.

SDS-PAGE was performed using 12% and 10% separating gel with 4% stacking gel for sarcoplasmic and myofibrillar proteins, respectively. Twenty microliters of protein were loaded onto the gel. Electrophoresis was performed at voltage of 80 V for 15 min, followed by an increased voltage to 120 V for 30 min. After separation, gels were immersed successively in fixative (10% acetic acid, 50% ethanol), stainer (0.25% coomassie brilliant blue R-250, 10% acetic acid, 50% ethanol) and destainer (8% acetic acid, 25% ethanol).

2.6. Total volatile basic nitrogen (TVB-N) contents

TVB-N was extracted and determined according to Fan, Luo, Yin, Bao, and Feng (2014). TVB-N contents were expressed as mg/100 g flesh.

2.7. Biogenic amines (BAs)

Five grams of muscle were homogenized with 10 mL $HClO_4$ (0.6 mol/L), and centrifuged at 10 000*g* for 5 min. The process was repeated once. All the obtained supernatant were combined and adjusted to 25 mL. The extract was stored at −18 ± 1 °C until analysis. After pre-column derivatization with dansyl chloride, the extract was separated on a COSMOSIL 5C18-PAQ column (4.6 mm × 250 mm), as reported by Fan et al. (2014). Putrescine and cadaverine were identified and quantified according to standards (Sigma-Aldrich Trading Co., Ltd., Shanghai, China).

2.8. Volatile organic compounds (VOCs)

Four grams of minced muscle were placed in a 20 mL glass vial sealed with crimp caps, and equilibrated in a water bath (40 °C) for 15 min. Then, a 65 μm Polydimethylsiloxane/Divinylbenzene (PDMS/DVB) fibre was exposed to the headspace of the vial for 40 min at 40 °C. The fibre was desorbed in the injector at 240 °C for 1 min in splitless