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Characterization of the microbiota in lightly salted bighead carp (*Aristichthys nobilis*) fillets stored at 4 °C

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ABSTRACT

The microbiota of unsalted and salted (dry-cured with 2% salt) bighead carp (*Aristichthys nobilis*) fillets during storage at 4 °C were identified by 16S rRNA gene analysis. Eleven genera were present in the initial microbiota of bighead carp fillets, where *Acinetobacter*, *Aeromonas* and *Kocuria* were the dominant bacteria. As storage time progressed, the microbial composition of both unsalted and salted fillets became less diverse. Additionally, differences in microbiota were observed between these two treatments. For unsalted bighead carp fillets, *Aeromonas* became the dominant genus at the end of storage and *Pseudomonas* was found less commonly. For salted fillets, *Pseudomonas* was the only bacteria identified at the end of storage.

1. Introduction

Bighead carp (*Aristichthys nobilis*) is a major freshwater fish species of aquaculture: 2,898,816 tons were harvested in 2012 (FAO, 2014). After death, bighead carp are highly prone to spoilage due to their rich nutrient content, which is utilized easily by bacteria (Zhu et al., 2012). Salting is a conventional method often used in the fish-processing industry to retard bacterial growth and extend shelf life of fish products. Additionally, as consumer preference for health food is growing, the demand for low-salt diets, such as lightly salted fish fillets, is increasing (Fan et al., 2014). Therefore, it is timely to study the effects of low levels of salt on fish quality.

Microbial growth is the major cause of fish spoilage. Freshly caught fish is contaminated naturally with various microbiota. During storage, these microbial populations may shift because of different abilities of the microorganisms to tolerate preservation conditions (Gram and Dalgaard, 2002). As a result, only a small fraction of microorganisms survive and contribute to fish spoilage, called “specific spoilage organisms” (SSOs; Dalgaard, 1995). These SSOs participate in the spoilage of fish by producing unpleasant off-flavors, causing discoloration or slime. Therefore, it is these SSOs that need to be inhibited for quality control purposes. To our knowledge, little information is available about the influence of light salting on the microbiota of fish products during storage.

The aim of this study was to investigate the effect of light salting on the microbial quality of bighead carp fillets and to characterize the dominant microbiota in both unsalted and salted bighead carp fillets by 16S rRNA gene analysis. Understanding the spoilage of unsalted and lightly salted bighead carp fillets can improve preservation methods that target SSOs and result in longer shelf life of related bighead carp products.

2. Materials and methods

2.1. Sample preparation

Farmed bighead carp (weight of 1445 ± 92 g, length of 49.5 ± 0.7 cm) were bought from a local aquatic products wholesale market in Beijing, China in July 2014, and transported to the laboratory alive. Within 2 h of arrival, the fish were stunned, scaled, gutted, filleted and washed with sterile water. Then fish fillets were divided into two groups: one unsalted group and one salted group that were sprinkled with 2% (w/w) salt. All samples were packed individually in polyvinyl chloride bags and stored in refrigerated room (4 ± 1 °C). Three randomly selected fillets were taken for

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analysis every two days, until they were deemed rejected by sensory evaluation.

2.2. Sensory analysis

Sensory analysis was conducted by an experienced nine-member sensory panel. Color, odor, morphology and elasticity were rated based on a five-point scale (5, excellent; 1, very poor). Scores of these four attributes were added to get an overall sensory score.

2.3. Total volatile base nitrogen (TVB-N)

Ten grams of minced sample was stirred in 100 mL of deionized water for 30 min, and then the mixture was filtered. The filtrate was used for the determination of TVB-N value, according to the semi-microtitration method of Zhu et al. (2012). The TVB-N value was expressed as mg/100 g flesh.

2.4. Biogenic amines (BAs)

Five grams of minced sample was homogenized twice with 10 mL of perchloric acid (0.6 M) for 1 min and centrifuged at 10, 000*g* at 4 °C for 15 min. The supernatants were combined and made up to 25 mL with 0.6 M perchloric acid. BAs were measured as dansyl chloride-positive substance following the method of Hong et al. (2013). Tryptamine, putrescine, cadaverine, histamine, tyramine, spermidine and spermine were identified and quantified using Sigma external standards.

2.5. Enumeration of different bacterial groups

Samples (0.1 ml) of serial dilutions (1:10, sterile 0.9% saline) of homogenates were spread on the surface of various microbiological media. Total aerobic bacteria (TAC) were cultivated on plate count agar (PCA), *Pseudomonas* sp. on *Pseudomonas* CFC selective agar, *Aeromonas* sp. on *Aeromonas* ampicillin selective agar, H_2S-producing bacteria on iron agar and lactic acid bacteria on MRS agar. All counts were expressed as $\log_{10}$ cfu g^{-1}.

2.6. Isolation and identification of the microbiota

Microbiota were analyzed initially, after 4 and 8 days of storage for the unsalted group, and after 8 and 12 days of storage for the salted group. After TAC enumeration, all colonies were selected from PCA plates that contained 30–100 colonies, cultivated in tryptic soy broth (TSB), and then streaked to purity on PCA. The purity of each isolate was verified by Gram staining.

The purified colony was cultivated in TSB for further amplification, after which 2 mL of TSB culture was centrifuged to form a cell pellet. Bacterial DNA was extracted according to the procedure for Bacterial Genomic DNA Extraction Kit (Bomad Biological Technology Co., Ltd., Beijing, China).

A fragment approximately 1400 bp of the bacterial 16S rDNA was amplified using universal primers: forward primer 27f (5′-GAGATTTGATCCTGGCTCAG-3′) (Parlapani et al., 2013) and reverse primer 1495r (5′-CTACGGCTACCTTGTTACGA-3′) (Hoti et al., 2003). The PCR mixture (25 μL) contained 12.5 μL 2 × Taq PCR Master Mix (DNA polymerase, dNTPs, $MgCl_2$, buffer), 10.5 μL double distilled water, 1 μL template DNA, and 0.5 μL of each primer (10 μM). Amplification was performed in a Gradient PCR TC-512 (Techne, UK) with the following conditions: 94 °C for 5 min; 35 cycles (94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min); and final extension at 72 °C for 10 min. All the reagents used for PCR reaction were supplied by BBT Co. Ltd (Beijing, China).

PCR products were submitted to BBT Co. Ltd (Beijing, China) for sequencing. A tentative identification was carried out by a similarity search using the Eztaxon (Chun et al., 2007) (http://www.eztaxon.org/). Sequences with more than 97% identity were recognized as the same species.

2.7. Statistical analysis

The least significant difference (LSD) procedure was used to test for difference between means (significance level was set at 5%) using SPSS 17.0 (SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Sensory analysis

The sensory score of 20 on day 0 indicated that all samples were of very excellent quality (Table 1). As storage time went on, freshness of both groups declined gradually. A fillet was considered completely spoiled when the sensory score reached 4. After being stored for 8 and 12 days, respectively, unsalted and salted samples became totally deteriorated.

3.2. Chemical analysis

TVB-N increased from an initial value (mg/100 g flesh) of 6.72 to 23.53 for unsalted fillets on day 8 and to 22.13 for salted fillets on day 12, which exceeded the acceptable TVB-N level (20 mg⁄100 g) for fresh freshwater fish (Table 1) (Song et al., 2011). Tyramine, spermidine and spermine were major amines for both groups and showed considerable fluctuations throughout storage (Table 2). Putrescine, which is used as a quality control index for many fish species (Shi et al., 2012; Fan et al., 2014), was first detected on days 4 and 8 for the unsalted and salted groups, respectively. At the end of storage, putrescine levels of unsalted and salted groups increased to 1.68 and 1.23 mg/kg, respectively. Histamine, the causative agent for fish poisoning, increased with time in both groups, but remained far below its tolerable limit (100 mg/kg flesh) (Kim et al., 2009). Tryptamine and cadaverine were not detected throughout storage. The amount and type of biogenic amines formed depends on the species of microbial flora present, species of fish and

Table 1
Changes in sensory scores and TVB-N values of unsalted and salted bighead carp fillets during storage at 4 °C.

		Storage time (day)						
		0	2	4	6	8	10	12
Sensory scores	unsalted	20.00 ± 0.00^{e}	17.50 ± 1.76^{d}	13.00 ± 1.26^{c}	9.50 ± 1.05^{b}	4.00 ± 0.00^{a}		
	salted	20.00 ± 0.00^{e}	19.83 ± 0.41^{e}	18.50 ± 0.84^{d}	13.67 ± 1.51^{c}	13.33 ± 0.52^{c}	8.17 ± 0.98^{b}	4.00 ± 0.00^{a}
TVB-N (mg/100 g)	unsalted	6.72 ± 0.56^{a}	8.68 ± 0.28^{b}	10.27 ± 0.86^{c}	11.30 ± 0.32^{c}	23.53 ± 0.84^{d}		
	salted	6.72 ± 0.56^{a}	8.40 ± 0.28^{b}	9.15 ± 0.32^{b}	10.18 ± 0.32^{c}	13.44 ± 0.28^{d}	14.47 ± 0.86^{e}	22.13 ± 0.28^{f}

Same lowercase letters in a row indicate no significant differences ($P > 0.05$).