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Short communication

Microbial succession of grass carp (*Ctenopharyngodon idellus*) filets during storage at 4 °C and its contribution to biogenic amines' formation

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ABSTRACT

Investigation on the microbial succession of grass carp filets during storage at 4 °C was carried out. For identification, 16S rRNA genes of the isolated pure strains were sequenced and analyzed. *Acinetobacter* was dominant in fresh grass carp. Species from the genera *Brevundimonas*, *Empedobacter*, *Pseudomonas*, *Microbacterium*, *Flavobacterium*, *Moraxella*, *Shewanella* and *Soonwooa* were also detected at the initial day. The communities were dominated by *Aeromonas* and *Acinetobacter* after 6 days. *Aeromonas* followed by *Pseudomonas* was the predominant genera at the end of shelf-life of grass carp, while other genera such as *Shewanella*, *Acinetobacter*, *Flavobacteriaceae* and *Psychrobacter* were present in smaller numbers. We investigated biogenic amines' (BAs) production by six strains isolated from spoiled grass carp filets. *Shewanella putrefaciens* showed significantly higher abilities to produce putrescine, than those from other genera. *Aeromonas veronii* revealed a strong ability to produce putrescine and cadaverine. However, *Pseudomonas* and *Acinetobacter* showed little ability to produce BAs.

1. Introduction

Grass carp (*Ctenopharyngodon idellus*) is one of the important freshwater species of aquaculture in China: 4,780,000 tons were produced in 2012 (Fishery Bureau of Ministry of Agriculture of the People's Republic of China, 2013). They are highly perishable commodities even under chilled storage. Microbial growth and metabolism are the major cause for food spoilage (Gram and Dalgaard, 2002). The organisms most commonly involved in freshwater fish are *Pseudomonas*, *Aeromonas*, *Flavobacterium*, *Shewanella*, *Micrococcus* and *Moraxella* (Austin, 2006; Chytiri et al., 2004; Mahmoud et al., 2004). The microbial populations may shift during storage and only a small fraction of fish microbiota is responsible for spoilage, known as "specific spoilage organisms" (Broekaert et al., 2013; Gram and Dalgaard, 2002). None of these studies have examined grass carp filets. It is meaningful to get information about microbial composition shift of grass carp during storage and it will provide an invaluable tool for storage management of grass carp under commercial conditions.

Biogenic amines (BAs) are mainly formed by microbial decarboxylation of amino acids and transamination of aldehyde and ketones in foods (Kim et al., 2009). They are important due to risk of food intoxication. In many published studies on BAs formation of fish, researchers have focused on the changes of BAs amount during storage and proposed biogenic amines as an indicator of quality deterioration (Li et al., 2012; Paleologos et al., 2004; Shi et al., 2012). Bacterial production of biogenic amines has seldom been studied in freshwater fish and little is known about such production in grass carp. Therefore, it is still important to investigate microbial species responsible for BAs formation in grass carp.

Different species in the same groups of bacteria may be differently affected by the same storage conditions (Pennacchia et al., 2011). Studying the microbial composition at the species-level is necessary. In most studies of microbiota associated with freshwater fish spoilage, the data were based on comparison of viable counts of different spoilage bacterial groups (Chytiri et al., 2004; Gui et al., 2013). The aim of this work was to carry out species-level survey of the microbial composition occurring on refrigerated grass carp filets under aerobic storage using culture dependent methods. The abilities of different microorganisms from spoiled grass carp filets to produce BAs were also discussed.

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2. Materials and methods

2.1. Samples

Grass carps (approx. 1500 g, the number is 12) from an aquatic product market (Beijing, China) were transferred to the laboratory alive in summer, 2013. The fish were stunned, scaled, eviscerated, filleted, and washed with cold sterile water immediately. After washing, the filets were drained in the constant temperature incubator at 4 °C for 10 min. Then the fish filets were packed in sealed polyvinyl chloride bags and stored in a refrigerator at 4 °C. Sensory evaluation, pH value, total volatile basic nitrogen (TVB-N), total viable counts (TVC) and biogenic amines (BAs) were measured at days 0, 2, 4, 6, 8, 10, and 12. Other microbiological analyses were carried out at days 0, 6 and 12. Three fish filets were randomly taken for each analysis time and each filet was obtained as one sample.

2.2. Sensory analysis

The raw filets were evaluated by six trained panelists. The sensory evaluation was based on a five point scale as described by Ojagh et al. (2010).

2.3. Determination of pH and TVB-N

The pH value was determined by a digital pH meter (FE20/EL20; Mettler Toledo, Shanghai, China) (Hong et al., 2012). TVB-N value was measured by the micro-diffusion method (Hong et al., 2012).

2.4. Microbial enumeration

Plate counts were carried out on samples to determine the total number of spoilage microorganisms. Twenty-five grams of fish meat was aseptically weighed and homogenized with 225 mL sterile 0.85% physiological saline for 1 min. Samples of serial dilutions were extracted following the methods described by Hong et al. (2013). Duplicate spread plates (100 μL) of each sample were spread on the surfaces of Plate Count Agar and incubated at 30 ± 1 °C for 72 h to calculate total viable counts (TVC). Duplicate spread plates of each sample (100 μL of serial dilutions) of *Aeromonas* Medium Base (AMB) were prepared for the enumeration of *Aeromonas* sp., and incubated at 30 ± 1 °C for 48 h. Duplicate spread plates of each sample (100 μL of serial dilutions) of *Pseudomonas* CFC Selective Agar (CFC), and Iron Agar were prepared for the enumeration of *Pseudomonas* sp., and H_2S producing bacteria, respectively, and incubated at 20 ± 1 °C for 48 h or 96 h for H_2S producing bacteria. All counts were expressed as log cfu/g.

2.5. Isolation and identification of microbiota

2.5.1. Isolation and purification

After TVC enumeration, bacteria from plate count agar plates were taken for isolation and identification of microbiota at 0, 6 and 12 days. For the bacteria purification, all of the isolates were picked from the highest dilution plates which usually contained 30–100 isolates, and subcultured at 30 ± 1 °C for 24–36 h in the nutrient broth. Isolates of each sample are purified on the surfaces of plate count agar at 30 ± 1 °C for 36–48 h. All of the purified isolates were observed for cell shape and Gram staining. All of the culture media were supplied by Hai Bo Biological Technology Co., Ltd. (Qingdao, China).

2.5.2. Identification of bacteria

A single colony was cultured in 5 mL of tryptic soy broth (TSB) (Aoboxing Universeen Bio-Tech Co., Ltd., Beijing, China) at 30 ± 1 °C for 18–24 h, and the cells from 2 mL of TSB culture were harvested by centrifugation. DNA was extracted from the sedimented cells by using the bacterial DNA isolation kit (Bomad Biological Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions. The extracts were assessed for quality by 1.0% agarose gel electrophoresis and used as DNA templates for further analysis.

To identify the single colony, the prepared genomic DNA was used as a template to amplify the bacterial 16S rDNA by PCR (TC-512, Techne, UK) which was performed with universal bacterial primers: forward primer 27f (5′-GAGATTTGATCCTGGCTCAG-3′) (Parlapani et al., 2013) and reverse primer 1495r (5′-CTACGGCTACCTTGTTACGA-3′) (Hoti et al., 2003). The primers were obtained from Bomad Biological Technology Co., Ltd. (Beijing, China; BBT). PCR system (final volume, 25 μL) contained 12.5 μL 2 × Taq PCR Master Mix (containing thermostable DNA polymerase, dNTPs, $MgCl_2$, buffer), 10.5 μL double distilled water, 1 μL of template DNA, and 0.5 μL of each primer at a concentration of 10 μM. The reagents were obtained from BBT Co., Ltd. (Beijing, China). PCR reaction consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles (denaturation at 94 °C, 30 s, primer annealing at 54 °C, 30 s, primer extension at 72 °C, 1 min), and a final extension step at 72 °C for 10 min. PCR amplification products (3 μL) were routinely checked on 1.0% agarose gels.

The sequence data were obtained from BBT Co., Ltd. (Beijing, China). The 16S partial sequences were mostly about 1400 bp. A tentative identification was performed by a similarity search using the Eztaxon (Chun et al., 2007) (http://www.eztaxon.org/). Sequences are considered to be of the same species if they group at greater than 97% similarity (Madigan et al., 2014).

2.6. Extraction and analysis of BAs

BAs from grass carp were extracted according to the method of Li et al. (2012). Five-gram fish mince was homogenized with 0.6 M cold (4 °C) perchloric acid (PCA; 10 mL) twice and centrifuged at 11,000 × *g* for 15 min. All supernatants were made up to 25 mL with 0.6 M PCA and stored at −20 °C for further analysis. Extraction of bacterial cultures was carried out according to the procedures developed by Kim et al. (2009) with some modification. The strains were cultured in 5 mL tryptic soy broth (TSB) with precursors: 0.5% L-histidine hydrochloride, L-lysine hydro-chloride, L-ornithine hydrochloride, and 0.25% L-tyrosine disodium salt supplemented with 0.0005% pyridoxal-HCl at 30 °C for 48 h. The reagents were obtained from Sigma. Bacterial culture (1 mL) was added to 4 mL 0.6 M cold (4 °C) PCA and homogenized. Then, broth cultures were extracted as described above.

The eight standard BAs, tryptamine (TRY), phenylethylamine (PHE), putrescine (PUT), cadaverine (CAD), histamine (HIM), tyramine (TYM), spermidine (SPD), and spermine (SPM) were purchased from Sigma-Aldrich (Shanghai, China). The eight standard BAs were mixed together at different concentrations.

Table 1
Changes in sensory scores, TVB-N and pH of grass carp filets during storage at 4 °C.

	Storage time (days)						
	0	2	4	6	8	10	12
Sensory scores	20 ± 0.00^{f}	16.83 ± 0.88^{e}	15.92 ± 1.74^{d}	13.00 ± 1.52^{c}	10.33 ± 1.89^{b}	8.33 ± 2.25^{b}	4.00 ± 0.00^{a}
TVB-N (mg/100 g)	6.54 ± 1.29^{c}	7.75 ± 1.71^{c}	9.80 ± 0.40^{bc}	9.52 ± 0.00^{c}	10.55 ± 1.97^{bc}	15.40 ± 4.88^{b}	27.82 ± 6.75^{a}
pH	6.89 ± 0.02^{c}	6.91 ± 0.02^{bc}	6.88 ± 0.04^{cd}	6.81 ± 0.04^{d}	6.92 ± 0.09^{bc}	6.99 ± 0.02^{b}	7.13 ± 0.05^{a}

[a] Same lowercase letters in a line indicate no significant differences ($p > 0.05$).