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# Spoilage potential of three different bacteria isolated from spoiled grass carp (*Ctenopharyngodon idellus*) fillets during storage at 4 °C

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ABSTRACT

In this study, the growth of *Aeromonas hydrophila*, *Pseudomonas jessenii* and *Shewanella putrefaciens* in sterile grass carp fillets stored at 4 °C was evaluated, as well as their effect on physicochemical and sensory spoilage. *A. hydrophila* and *P. jessenii* strains increased rapidly during the first 10 days, but *S. putrefaciens* showed a lag phase. The highest producers of total volatile basic nitrogen were *A. hydrophila* and *P. jessenii*, which reached a maximum level around 30 mg/100 g after 16 days. *P. jessenii* was an active producer of biogenic amines in grass carp fillets and was the only bacterium that produced histamine among the three bacteria. All the inoculated samples produced abundant cadaverine, which reached 51.5, 54.8 and 37.8 mg/mL for *A. hydrophila*, *P. jessenii*, and *S. putrefaciens*, respectively. The influence of bacteria on the thiobarbituric acid reactive substances (TBARS) value of grass carp fillets was slight. Volatile compounds, such as tetradecanal and 1-hexadecanol, were detectable only for *S. putrefaciens*, which also produced the highest amount of 1-octen-3-ol, nonanal and 2-ethylcyclohexanol. *P. jessenii* produced high levels of ketones such as 2-nonanone and 2-undecanone.


## 1. Introduction

Grass carp (*Ctenopharyngodon idellus*) is one of the most important commercial freshwater fish species in the world. The world aquaculture production of grass carp was 5,537,794 tons in 2014 (FAO, 2016, p.30). They are highly perishable, even under chilled storage. Generally, initial deterioration of quality of fresh fish is caused primarily by autolytic changes, whereas subsequent loss of quality and spoilage occur as a result of bacteriological activity (Katikou, Georgantelis, Paleologos, Ambrosiadis, & Kontominas, 2006). Biogenic amines (BAs) are basic nitrogenous compounds, most of which are present in fresh grass carp at very low levels. The gradual accumulation of BAs, such as tryptamine, 2-phenylethylamine, putrescine, and cadaverine are associated with the growth of bacteria (Wang et al., 2014). Although, much is known about the relationship between bacteria and the accumulation of BAs, little is known about the microbial species responsible for the formation of BAs in freshwater fish (Gui, Binzhao, Song, Zhang, Hui, & Li, 2013; Li, Bao, Luo, Shen, & Shi, 2012; Shi, Cui, Lu, Shen, & Luo, 2012). Moreover, because the total volatile basic nitrogen (TVB-N) is a common indicator of the freshness of freshwater fish (Zhang, Shen, & Luo, 2011), we investigated the production of TVB-N by different spoilage bacteria.

*Acinetobacter*, *Pseudomonas*, *Aeromonas*, *Flavobacterium*, *Shewanella*, *Micrococcus*, *Carnobacterium* and *Moraxella* are the most common organisms cultured from freshwater fish (Austin, 2006; Gui et al., 2013; Y.; Zhang, Li, Li, Liu, & Luo, 2015). In our previous study, *Aeromonas, Pseudomonas* and *Shewanella* were identified to be the major spoilage flora in spoiled grass carp fillets (Wang, Luo, Huang, & Xu, 2014). At the onset of spoilage, only specific spoilage organisms participate in the spoilage process and produce metabolites that result in off-odors and off-flavors (Gram & Huss, 1996). Ammonia-like off-odors and sour off-flavours were found in spoiled chilled seafood products. *Shewanella putrefaciens* is a well-known spoilage bacterium of marine fish that produces trimethylamine and $H_2S$, which lead to intensive off-odors (Dalgaard, 1995; Macé, Cardinal, Jaffrès, Cornet, Lalanne, Chevalier, et al., 2014; Paarup et al., 2002). However, no clear link has been

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established between spoilage characteristics and specific spoilage organisms in freshwater fish.

In this study, sterile grass carp fillets were inoculated with the monocultures of three potential spoilage bacteria: *Aeromonas hydrophila*, *Pseudomonas jessenii* and *Shewanella putrefaciens*. All of the bacterial groups were isolated previously from spoiled grass carp that was stored at chilled (4 °C) temperature. The aim of this study was to investigate the spoilage potential of these three bacteria during storage. The study was based on measurements of total viable counts, sensory evaluation, TVB-N, the thiobarbituric acid reactive substances (TBARS), BAs and volatile compounds of the inoculated fish fillets, compared to non-inoculated control.

## 2. Materials and methods

### 2.1. Bacterial isolates

All the strains used in this study were isolated previously from spoiled grass carp and maintained as frozen stocks at −80 °C in a cryoprotective medium containing, which contained 10% skim milk powder. The strains were identified by 16S rRNA gene partial sequencing and belonged to the taxonomic groups of *Aeromonas hydrophila*, *Pseudomonas jessenii* and *Shewanella putrefaciens*. Five strains that belong to the each specie were chosen. All the isolates of *Aeromonas hydrophila*, *Pseudomonas jessenii* and 4 of *Shewanella putrefaciens* were isolated from spoiled grass carp in our previous study (Wang, Luo, Huang et al., 2014), and another strain of *Shewanella putrefaciens* was isolated and identified from the spoiled grass carp in another previous study (no publications).

### 2.2. Sampling

#### 2.2.1. Sterile fish fillets

Fifty grass carp were purchased from an aquatic product market in Beijing, China in April 2014 and transported to the laboratory alive in aerated foam boxes that contained water. The mean weight and length of fish were about 1500 g and 36 cm. The sterile grass carp fillets were obtained by the method described by Mace et al. (2013), with some modification. Grass carp were killed, scaled, gutted, and washed under running water. After washing, the skin surface of the fish was wiped with a 75% alcohol solution. Next, the muscle from the backbone of the fish was hand-filleted, skinned, and cut into fish fillets (about 2 × 4 × 5 cm). Each fish fillet weighed between 15 and 20 g and only white muscle was collected. The fillets were rinsed with sterile water and then soaked in 2% formalin for 30 s (about 48 fillets in 1 L of 2% formalin). Finally, the fillets were soaked in sterile water and washed three times. All fillets were aired under the ultraviolet radiator in a clean bench (Yatai Kelong, Co. Ltd, Beijing, China) for 30 min. The ultraviolet radiator was used to make sure the sterile fish fillets were aired under the sterile environment in order to avoid bacterial contamination. The initial total viable counts (TVC) of the sterile fish fillets were below 1.0 log cfu/g. It was cost about 1.5 h from purchasing to postmortem. All the samples were prepared within 2 h after postmortem and the fish fillets were kept on the ice during sampling process.

#### 2.2.2. Sample inoculation and packaging

The strains were pre-cultured individually in a tryptic soy broth (TSB) at 30 °C for 24 h (Aoboxing Universeen Bio-Tech CO., LTD. Beijing, China). Each culture (200 μL) was mixed with 10 mL TSB in a flask and cultured in a concussion incubator at 30 °C for 8–10 h to achieve a level of approx. 10.0 log cfu/mL. The culture was then diluted 1000-fold in a sterile saline solution (0.9% NaCl) in order to achieve an inoculation mixture that contained 7.0 log cfu/mL. Cultures of five strains that belong to the same species were mixed together in a sterile conical flask. The five strains were mixed together to avoid the strain effect, in which strains that belong to the same species can present a heterogeneous spoilage potential (Stohr, Joffraud, Cardinal, & Leroi, 2001). The sterile fish fillets were soaked in the each bacterium suspension (about 48 fillets in 1 L of bacterium suspension) for 10 min and reached an inoculated level of 3 log cfu/g. A control was prepared by inoculating sterile fillets with sterile water instead of the strain mixture. After the inoculation, every three or four of the inoculated fish fillets were packaged randomly in sterile polyvinyl chloride bags and stored in a refrigerator at 4 °C. TVC, sensory evaluation, TVB-N, and drip loss were measured every two days (on days 0, 2, 4, 6, 8, 10, 12, 14, and 16). BAs and TBARS were measured every four days (on days 0, 4, 8, 12, and 16). The volatile compounds that were produced by the inoculated samples and the non-inoculated control samples were analyzed on day 0 and day 16. Three packages of fish fillets were selected randomly for every treated group at each storage time. Each package was treated as one sample. At each storage time, all the measurements were performed on the same package.

### 2.3. Microbiological analysis

Plate counts were carried out on samples to determine the total number of spoilage microorganisms. Five grams (5 g) of grass carp flesh was transferred to sterile stomacher bags with 45 mL sterile saline solution and homogenized for 15 s with a masticator (Masticator Basic L, S.A. Spain). The homogenized samples were serially diluted (1:10) serially in sterile saline solution. Samples of serial dilutions (100 μL) were spread on the surfaces of Plate Count Agar (Hai Bo Biological Technology Co. Ltd, Qingdao, China) and incubated at 30 ± 1 °C for 72 ± 3 h to calculate TVC. All counts were expressed as log cfu/g.

### 2.4. Sensory analysis

Six trained panelists, who were experienced in sensory evaluation of freshwater fish, conducted the sensory analysis of the grass carp fillets according to a conventional profile (ISO, International Organization for Standardization, 2012). The panelists were required to score the spoilage level of the grass carp fillets on a continuous scale from 0 to 10 (Mace et al., 2013).

### 2.5. Chemical analysis

#### 2.5.1. Total volatile basic nitrogen (TVB-N)

TVB-N of the samples was measured by the method described by Song, Liu, Shen, You, and Luo (2011). Ten grams (10 g) of fish flesh was dispersed in 100 mL distilled water and stirred for 30 min. The mixture was run through filter paper and collected. A microtitration method was used to analyze TVB-N with Kjeldahl Apparatus (KDY-9820, Beijing, China). TVB-N value was expressed as mg TVB-N per 100 g muscle.

#### 2.5.2. Thiobarbituric acid reactive substances (TBARS)

TBARS were determined according to Erkan and Özden (2008). The values of the TBARS were expressed as milligram of malonaldehyde equivalents per kilogram of fish flesh.

#### 2.5.3. Centrifugation loss

Centrifugation loss was measured as described by Hultmann and Rustad (2002). Three grams (3 g) ($W_a$) of fish flesh was weighed and packed with filter paper in centrifuge tube. Samples were centrifuged at 1760 *g* for 5 min at 4 °C and then weighed again ($W_b$). The centrifugation loss (%) was expressed as $100 \times (W_a - W_b)/$