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Inhibition of microbial spoilage of grass carp (*Ctenopharyngodon idellus*) fillets with a chitosan-based coating during refrigerated storage

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Inosine monophosphate (PubChem CID: 8582)
Hypoxanthine ribonucleoside (PubChem CID: 6021)
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Trimethylamine (PubChem CID: 1146)

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ABSTRACT

The effects of a chitosan-based coating on the inhibition of microbial spoilage of grass carp (*Ctenopharyngodon idellus*) fillets were studied during refrigerated storage for 15 days in terms of pH, total volatile base nitrogen (TVB-N), trimethylamine (TMA), adenosine triphosphate (ATP)-related compounds, K value, microbial enumeration and high-throughput sequencing. The results indicated that the fillets treated with chitosan-based coating enriched with 0.3% glycerol monolaurate and 0.5% clove essential oil had lower values of TVB-N, TMA, hypoxanthine riboside (HxR), hypoxanthine (Hx) and K value with the significant reductions ($P < 0.05$) of nearly 34, 73, 32, 74 and 38%, respectively, when compared to the control at day 15 of storage. Using high-throughput sequencing analysis, the major bacteria phyla of *Firmicutes*, *Cyanobacteria* and *Bacteroidetes* and the bacteria family of *Lachnospiraceae* and *Bacteroidaceae* were observed in fresh grass carp. As storage time increased, the coated samples retained bacterial diversity. However, *Shewanellaceae*, *Pseudomonadaceae* and *Flavobacteriaceae* increased and became the predominant microbiota in spoiled control samples. The significant difference between the bacteria species in the control and coated samples showed that the coating had the potential to inhibit microbial growth, especially spoilage microorganisms, and reduced quality deterioration caused by bacteria during refrigerated storage of grass carp fillets.

1. Introduction

China is the world's largest producer of freshwater fish and grass carp (*Ctenopharyngodon idellus*) is one of the most popular species. According to the China Fishery Yearbook, nearly 5.9 million tonnes were produced in 2016, making it the number one cultured fish in China (Anonymous, 2016). Recently, with rapid developments of reliable cold-chain, online shopping and consumer preference, the sale of preprocessed fresh fillets is increasing due to its convenience for subsequent processing. However, fresh fillets are highly perishable even with refrigeration (Liu et al., 2013; Zhang et al., 2011). Despite enzyme activities and lipid oxidation, microbial activity is often the main cause of spoilage through the accumulation of deleterious substances and unpleasant off-odors (López de Lacey et al., 2014). Psychrophilic bacteria such as *Pseudomonas*, *Shewanella* and *Aeromonas* are the dominant microbiota in chilled fishery products and are involved in spoilage (Parlapani et al., 2014). Wang et al. (2014) indicated that *Shewanella* and *Aeromonas* were major producers of putrescine and cadaverine. D Li et al. (2017) reported that the breakdown of inosine monophosphate (IMP) to HxR and Hx was mainly due to microbial activity. In addition, TMA, which was one of main compounds responsible for fishy odor, was also produced by microbial metabolism (Gram and Dalgaard, 2002). Therefore, maximizing the short shelf life of fresh fillets by inhibiting the growth of spoilage microorganisms remains an important need.

Recently, edible coating technology has been perceived as an effective and eco-friendly way to maintain the quality and safety of food products during storage (Guo et al., 2015; Kerch, 2015). Many edible coating materials such as chitosan, gelatin, alginate and whey proteins have been shown to have antibacterial activity that helps extend the shelf life of aquatic products when they were used alone or with natural preservatives. Souza et al. (2010) and Mohan et al. (2012) indicated that chitosan coatings reduced the total viable count (TVC) and extended the shelf life by 3–5 days for chilled salmon (*Salmo salar*) and

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sardine (*Sardinella longiceps*) fillets. Wang et al. (2017) showed that collagen coatings combined with lysozyme was effective to maintain the quality of refrigerated fresh salmon fillets. Shokri and Ehsani (2017) also showed that whey protein coatings enriched with lactoperoxide and α-tocopherol effectively inhibited bacterial growth (including TVC, psychrotrophs, *P. fluorescens* and *S. putrefaciens*) and extended the shelf life of pike-perch (*S. lucioperca*) fillets. However, most of early studies have only focused on the effect of edible coatings on the quality of aquatic products in terms of the observing bacterial enumeration, physicochemical changes and sensory scores. Information about how edible coatings affect microbial composition during storage is limited due to the inability to enumerate > 1% of the total bacterial population when using traditional microbial plate count methods (Ward et al., 1990), and this information is meaningful for exploring the reasons of coating preservation.

At present, some studies used glycerol monolaurate (GML) or clove essential oil (EO) as antibacterial agents in chitosan coatings and confirmed their synergistic effect to inhibit the growth of TVC and spoilage organisms using bacterial enumeration (XP Li et al., 2017; Yu et al., 2017). Besides, our previous study further showed the positive effect of chitosan coatings enriched with GML and EO on flavor quality preservation of fish (Yu et al., 2018). However, information about how the coating treatment affects microbial composition in fillets during refrigerated storage is limited. Thus, the purpose of this study was to evaluate the effects of chitosan-based coating on bacteria-induced biochemical quality changes in term of pH, TVB-N, TMA, ATP-related compounds and K value along with a combination of high-throughput sequencing and microbial enumeration to determine the effects of the coating treatment on bacterial composition and dominant microbiota in grass carp fillets with refrigeration.

2. Materials and methods

2.1. Coating solution and sample preparations

Chitosan powder extracted from crabs (85% degree of deacetylation and 400 kDa average Mw) was purchased from Haidebei Marine Bioengineering Co. (Jinan, Shandong, China). Glycerol monolaurate (GML) and clove essential oil (EO) were purchased from Hangzhou Funchun Food Additive Co., Ltd. (Hangzhou, Zhejiang, China) and Tiamay Aromatic Plant Co. (Shanghai, China), respectively. A 2% chitosan solution was prepared in 1% acetic acid (v/v). The solution also included glycerol (0.25 g/g chitosan) as a plasticizer. Then, the GML solution (GML: ethanol: Tween 20 = 1:1:6) mixed with EO was prepared and dispersed evenly in the chitosan solution using the method described by Yu et al. (2018). The final concentrations of GML and EO in solution were 0.3 and 0.5%, respectively. After that, the solution was used immediately for coating. Besides, a preliminary study was conducted to determine the dipping solution for control group. Result indicated that acidic acid solution dipping had no positive effect on quality retention of fillets, but it affected surface color of fillets and reduced the sensory quality. Therefore, distilled water was used as dipping solution for control group.

A total of 24 live grass carps, with an average weight of 2.5 ± 0.2 kg, were obtained from local supermarket (Wuxi, Jiangsu, China) in May 2017. After being slaughtered using percussive stunning by trained personnel, the carps covered with crushed ice were transported to the laboratory within 20 min. Subsequently, these carps were scaled, decapitated, eviscerated, filleted and skinned, followed by washing with tap water. The dorsal muscle of each fillet without skin were cut into cubes (~4 cm × 3 cm × 1.5 cm) as small fillets and then washed with cold (4 °C) sterile distilled water. Afterwards, the fillets were left to drain for 20 min in a sterile biochemical incubator (4 °C and 50% RH, Shanghai Yiheng Instruments Co., Ltd., Shanghai, China). All samples were divided randomly into two groups: one was immersed in distilled water as the control group (CK) and the remaining fillets were immersed in the edible chitosan-based solution (CT), with a sample/solution ratio of nearly 1:5 (m/v). After immersion for 5 min, all samples were dried in the sterile incubator (4 °C and 50% RH) for 90 min. Finally, each fillet was individually packed in a sterile polyethylene bag and stored at 4 ± 0.5 °C. The sampling time from each group for biochemical and microbial counting analyses was on 1st, 3rd, 7th 11th and 15th day of storage, while high-throughput sequencing was on 0th (defined as fresh sample), 7th and 15th day of storage.

2.2. pH

pH of samples was measured using the Chinese standard (GB/T 5009.24-2003). Five grams of sample were homogenized with 45 mL of cold distilled water for 1 min using a T10 shear mixer (IKA Werke GmbH & Co. KG, Staufen, Germany). The pH value was determined using a digital pH meter (Mettler Toledo EL20, Shanghai, China).

2.3. TVB-N and TMA

TVB-N (mg N/100 g sample) was measured using the micro-diffusion method. A distilling apparatus (KDN-103F, Shanghai Qianjian Instrument Co., Ltd., Shanghai, China) was used to collected distillate and the result was calculated by the titration volume of a 0.01 M HCl standard solution. TMA (mg N/100 g sample) was measured using AOAC (1980) method 971.14. The absorbance was read using a UV-1000 spectrophotometer (Techcomp Co., Ltd., Shanghai, China) at 410 nm against reagent blank. TMA (purity > 98%, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) was used to prepare the standard curve and then calculate TMA values in samples.

2.4. ATP-related compounds

ATP-related compounds (μmol/g sample) in samples were extracted with 6% (v/v) perchloric acid solution and analyzed using HPLC equipment according to the method used by Yu et al. (2018). The nucleotide standards including adenosine triphosphate (ATP, ≥ 98.5%), adenosine diphosphate (ADP, ≥ 95%), adenosine monophosphate (AMP, ≥ 99%), inosine monophosphate (IMP, ≥ 98%), hypoxanthine riboside (HxR, ≥ 99%) and hypoxanthine (Hx, ≥ 98%) were purchased from Sigma-Aldrich Co. (Shanghai, China). The concentration of the ATP-related compounds was based on the peak area comparisons with their respective standard using a Waters e2695 HPLC (Milford, MA, USA) equipped with a Waters 2998 detector and a Waters C_{18} column (5 μm, 4.6 mm id × 250 mm). The mobile phases were 0.05 M phosphate buffer (KH_2PO_4 and K_2HPO_4, pH 6.8) and methanol (98:2, v/v), with a flow rate of 1 mL/min. Detection wavelength and column temperature were 254 nm and 30 °C, respectively. Similar recovery rates of ATP-related compounds in control and coated samples were detected, with the ranges of 91.4–93.6%, 86.6–91.5%, 89.2–94.1%, 93.4–95.3%, 89.8–96.9% and 92.4–95.2% in ATP, ADP, AMP, IMP, HxR and Hx, respectively, indicating that the method had good reproducibility. K value was calculated by the following equation:

$$\text{K value (\%)} = [(\text{HxR} + \text{Hx})/(\text{ATP} + \text{ADP} + \text{AMP} + \text{IMP} + \text{HxR} + \text{Hx})] \times 100$$

2.5. Enumeration of microbial communities

Samples of fish muscle (10 g) were aseptically weighted and transferred to Stomacher bag (Turelab, Shanghai, China) with 90 mL sterile 0.85% NaCl and 0.1% peptone water. The mixture was fully homogenized using a Stomacher (BW-400P, Turelab), and the decimal dilutions of homogenates were prepared for bacterial determinations using plate pouring method. Total viable counts (TVC) were determined using plate count agar and they were incubated at 30 °C for 3 days.