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Effects of pomegranate peel extract on quality and microbiota composition of bighead carp (*Aristichthys nobilis*) fillets during chilled storage

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ABSTRACT

This study aimed to investigate effects of aqueous pomegranate peel extract (APPE) and ethanolic pomegranate peel extract (EPPE) on microbiota and changes in quality of bighead carp (*Aristichthys nobilis*) fillets stored at 4 °C. The results showed that pomegranate peel extract (PPE, which includes both APPE and EPPE) retarded the deterioration of sensory quality and flesh color, inhibited the growth of spoilage bacteria, and attenuated the production of biogenic amines, total volatile basic nitrogen (TVB-N), and the degradation of ATP-related compounds. Moreover, EPPE performed better in color attributes and biogenic amines, but APPE was more effective in retarding the increase of TVB-N and K-value. High-throughput sequencing results showed that microbial composition of all samples became less diverse as storage time increased. For the control group, *Acinetobacter* was predominant in the middle-period of storage, while *Pseudomonas*, *Aeromonas*, and *Shewanella* became predominant at the end of storage. Additionally, PPE decreased the relative abundance of *Acinetobacter* in the middle-period of storage, and thus changed the microbial composition. Based on our assessments of quality and microbial analysis, PPE prolonged the shelf-life of bighead carp fillets for about 2 days, and it has the potential to become a promising preservative in aquatic products.

1. Introduction

As one of the four major Chinese carp, bighead carp (*Aristichthys nobilis*) is popular with Chinese consumers for its high nutritional value and delicious taste. The Chinese aquaculture production of bighead carp was 3,097,952 tons in 2017, and it ranked fourth highest in production among freshwater fish species in China (Ministry of Agriculture and Fisheries Bureau., 2018). However, fresh fish are highly perishable, because their pH, water content, and nutrient richness constitute conditions favorable to microbial growth (Olatunde et al., 2018; Viji et al., 2017). Microorganisms are the main cause of fish spoilage, which is due to their uncontrolled growth and vigorous metabolisms (Chaillou et al., 2015; Huang et al., 2017; Sallam et al., 2007). For these reasons, inhibiting microbial growth can be vital for preserving fish products.

To retard the deterioration of fresh fish, chemical compounds like chlorine dioxide have been widely used for a long time. However, the use of synthetic compounds in food preservation is becoming limited with increasing concerns about food safety (Olatunde et al., 2018; Viji et al., 2017). Due to potential toxicity of chemical additives, an increasing number of studies have focused on natural phytochemicals, such as plant-derived essential oils and polyphenols, as food preservatives (Huang et al., 2017; Lytou et al., 2018; Yuan et al., 2016).

Pomegranate peel is a byproduct produced during the processing of pomegranate juice (Gullon et al., 2016). Pomegranate peel extract (PPE) has excellent antioxidant properties and antimicrobial activities due to its high content of phenolic compounds that include punicalagin, punicalin, gallic acid, and ellagic acid (Akhtar et al., 2015; Amyrgialaki et al., 2014; Gullon et al., 2016; Pagliarulo et al., 2016; Wafa et al., 2017). Many researchers have studied the preservative effects of PPE on chicken and beef products (Kanatt et al., 2010; Lytou et al., 2018; Turgut et al., 2017) and its antioxidant effects on fish flesh during frozen storage (Berizi et al., 2016, 2018; Özalp Özen and Soyer., 2018). However, the effects of pure PPE on the quality of refrigerated freshwater fish are poorly understood, especially the influence of PPE on the bacterial community in fish flesh. Moreover, the effects of PPE on the quality and microbiota composition of fish flesh can be affected by the chemical components in PPE. The components in PPE can be altered by the ratio of water/ethanol, both of which are efficient extraction agents

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in the preparation of PPE (Amyrgialaki at al., 2014; Wang et al., 2013; Zarei et al., 2015). However, it is unclear if there is any difference in preservative effects of aqueous pomegranate peel extract (APPE) and ethanolic pomegranate peel extract (EPPE) on fish flesh.

Therefore, this study aimed to assess preservative effects of APPE and EPPE on bighead carp fillets stored at 4 °C in terms of total volatile basic nitrogen (TVB-N), biogenic amines, ATP-related compounds, color, and sensory scores. We also characterized changes of bacterial communities in samples during chilled storage by microbial enumeration and high-throughput sequencing, because the study of microbial composition is significant for the understanding of fish spoilage process and the evaluating of preservative effects.

2. Materials and methods

2.1. Preparation of pomegranate peel extract

The preparation of PPE (i.e., including both APPE and EPPE) followed the traditional solvent extraction method of Amyrgialaki et al. (2014), with some modifications. Ripe pomegranate (*Punica granatum* L.) fruits (620 ± 105 g) were harvested in Huili County, Sichuan Province. The peels were separated manually from the washed fruits. Subsequently, 200 g blended peels were homogenized and mixed with 1000 mL acidified water (adjusted to pH 2.0 ± 0.1 with hydrochloric acid) and the mixture was stirred for 1 h at 25 °C in the dark. Afterwards, the extraction mixture was filtered through a filter cloth of 100 meshes and subjected to a rotary evaporator at 45 °C to remove most of the solvent. The concentrated solution was then centrifuged at 7000 g for 5 min, and the resulting yellow and transparent supernatant was designated as APPE. EPPE was prepared in the same way as APPE, but we used acidified ethanol solution (ethanol/water = 1.0, (v/v)) as the extraction agent. Ethanol was removed by rotary evaporation.

2.2. Determination of concentration of total phenolic compounds in PPE

Determination of concentration of total phenolic compounds in PPE was performed according to a modified Folin-Ciocalteu colorimetric method (Folin and Ciocalteu, 1927; Sun et al., 2013). Briefly, 0.08 mL sample was mixed well with 0.20 mL 1 M Folin-Ciocalteu reagent and 1.6 mL of 7.5% sodium carbonate solution. The mixture was kept for 1 h at 25 °C in the dark, and the absorbance was measured using a spectrophotometer (Unico Instrument Co. Ltd, Shanghai, China) at 765 nm. Standard gallic acid solution within the 0.05–0.25 mg/mL concentration range was used to establish a calibration curve. The concentration of total phenolic compounds was expressed as mg gallic acid equivalent/mL of sample (mg GAE/mL). Based on the determined concentration of total phenolic compounds, both APPE and EPPE were diluted to the same concentration (0.5 mg GAE/mL) with sterile water, and their pH was adjusted to 6.0 ± 0.1 with 0.01 M NaOH solution.

2.3. Preparation of fish samples

Farmed bighead carp (weight of 1.55 ± 0.15 kg, length of 49.6 ± 2.1 cm) were purchased from a local aquatic products market and transported to the laboratory alive in closed polyethylene bags with clear water (15 °C) and oxygen. After a temporary holding in clean water for 12 h, bighead carp were stunned by mechanical percussion which involves one or two sharp blows on the carp's head with a wooden club. Then, bighead carp were scaled and gutted. The percussive stunning method used in our experiment was a method recommended by World Organisation for Animal Health. (2012). The dorsal flesh of fish was filleted for our study, followed by washing with cold sterile water. Afterwards, the fillets (12 × 4 × 2 cm, 100 ± 10 g) were left to drain for 10 min and then divided into three groups randomly. About 2400 g fish fillets of control group, APPE group, and EPPE group were treated by being immersed in sterile water (3600 mL), APPE solution (3600 mL, 0.5 mg GAE/mL), and EPPE solution (3600 mL, 0.5 mg GAE/mL) for 10 min, respectively. Then, fillets were aerobic-packaged individually in high-density polyethylene (HDPL) bags with some air and stored in a refrigerator (4 °C). Three samples of each group were selected randomly for quality and microbiota analysis during storage.

2.4. Sensory analysis

Sensory analysis was performed by a six-member experienced sensory panel (three females and three males, 20–30 years old), according to the method of Berizi et al. (2016), with some modifications. The sensory characteristics of each fillet were evaluated for color, odor, texture, and overall acceptability using the following 5-point scale. A score of 4.0–5.0 indicated good quality, 3.0–4.0 indicated acceptable quality, 2.0–3.0 indicated unacceptable quality, and 1.0–2.0 indicated an intense dislike. Thus, 3.0 was the division between sensory acceptance and sensory unacceptance.

2.5. Measurement of color and TVB-N

The color measurement was performed by using a chroma meter (Model No. CR-400, Konica Minolta Sensing Inc., Tokyo, Japan), according to the method of Huang et al. (2017). L* (lightness), a*(redness/greenness), and b* (yellowness/blueness) values of the flesh were measured in our study. To represent the color of flesh accurately, three locations in the white muscle of each fillet were analyzed. The value of TVB-N was determined based on the micro-titration method (Song et al., 2011) with minor modification. Fish flesh (5 g) was homogenized with 50 mL of deionized water, and then the mixture was stirred at 100 r/min for 30 min. After a centrifugation (4500 r/min for 3 min), 5 mL of the resulting supernatant was well mixed with 5 mL MgO suspension (10 g/L), and then the mixture of the supernatant and MgO was to distill through Kjeldahl Apparatus (KDY-9820, Beijing, China). Meanwhile, 10 mL of 2% boric acid solution containing a mixed indicator produced from methyl red and methylene blue was used to absorb the distillate. After that, the boric acid solution was titrated with 0.01 M hydrochloric acid solution. TVB-N value was determined and expressed as mg N/100 g flesh, according to the consumption of hydrochloric acid.

2.6. Determination of ATP-related compounds and K-value

The extraction and HPLC analysis of ATP-related compounds was carried out according to the method of Vázquez-Ortiz et al. (1997) with some modifications. Briefly, 1 g sample of fish muscle was homogenized with 2 mL of 10% cold perchloric acid (PCA) solution and centrifuged at 1500 g for 3 min at 4 °C. The supernatant was collected while the obtained sediment was washed with 2 mL of 5% cold PCA solution and centrifuged at the condition described above. This process was repeated again. Afterwards, all supernatants were pooled together, and adjusted to pH 6.35–6.45 with 10 M and 1 M potassium hydroxide solutions. Potassium perchlorate formed in pH adjusting was removed by a centrifugation at 1500g for 3 min. The supernatant was diluted to 10 mL with 5% neutral PCA solution (adjusted to pH 6.4 by 25% ammonia), and then stored at −20 °C for HPLC analysis.

Then, the extracts of ATP-related compounds were analyzed using a HPLC equipped with a COSMOSIL 5C18-PAQ column (4.6 ID × 250 mm × 5 μm) and an SPD-10 A (V) detector set at 254 nm. ATP-related compounds were eluted using 0.05 M phosphate buffer (pH 6.8) at a flow rate of 1 mL/min. The injection volume was 50 μL and the elution was performed at 25 °C. K-value was calculated according to the following formula: K-value (%) = [(HxR + Hx)/(ATP + ADP + AMP + IMP + HxR + Hx)] × 100. where HxR is hypoxanthine riboside, and Hx is hypoxanthine.

2.7. Determination of biogenic amines

The extraction of biogenic amines were carried out according to the method of Liu et al. (2017). Five grams of white muscle was homogenized with 10 mL of 0.6 M cold perchloric acid (PCA) solution and centrifuged at 10,000 × g for 5 min. The supernatant was collected, while the pellet was washed and centrifuged again. The two supernatants were pooled and adjusted to 25 mL with 0.6 M cold PCA. The samples were stored at −20 °C for further analysis. Derivatization and analysis of biogenic amines were conducted according to the HPLC method of Hong et al. (2013) and Smela et al. (2003). Briefly, 0.4 mL of biogenic amines extract was mixed with 80 μL 2 M NaOH and 120 μL saturated $NaHCO_3$ solution, followed by 0.8 mL of 10 mg/mL dansyl chloride dissolved in acetone. Then, the mixture was incubated at 40 °C for 45 min and stopped by adding 40 μL 25% ammonia. After keeping for 30 min at 25 °C, the mixture was diluted to 2 mL with acetonitrile. Then, biogenic amines was quantified using a HPLC equipped with a COSMOSIL 5C18-PAQ column (4.6 ID × 250 mm × 5 μm) and a SPD-10 A (V) detector set at 254 nm. Biogenic amines were eluted using ammonium acetate (0.1 M; solvent A) and acetonitrile (solvent B) as mobile phases, at a flow rate of 0.8 mL/min. The elution gradient is: 0 min, 50% A; 25 min, 10% A; 35 min, 10% A; 45 min, 50% A. The sample was eluted at 30 °C with an injection volume of 10 μL. Biogenic amines were determined and quantified based on the retention time and peak area compared with standard solutions.

2.8. Microbial enumeration

Enumeration of total viable microorganisms and selective culture of *Pseudomonas*, *Aeromonas,* and H_2S-producing bacteria were carried out according to the method described by Huang et al. (2017). Briefly, 5 g of fish sample was homogenized with 45 mL sterile physiological saline in a Stomacher (Masticator Basic L, S.A. Spain) to produce the 1st dilution. Spread plating method (0.1 mL of serial 10-fold dilutions in physiological saline) was used for microbial enumeration. H_2S-producing bacteria and *Pseudomonas* were aerobically incubated at 25 °C for 48 h, using iron agar medium and *Pseudomonas* agar (Oxoid code CM0559, U.K.) added with *Pseudomonas* CFC supplement (SR 0103E), respectively. Counts of total viable microorganisms and *Aeromonas* were determined using plate count agar and *Aeromonas* medium base (Oxoid code CM0833, U.K.) supplemented with SR 0136E, respectively, after aerobic incubation at 30 °C for 48 h.

2.9. High-throughput sequencing

Ten grams of flesh cut off from various parts of one fillet was mixed with 20 mL sterile physiological saline, and then shaken on a gyratory shaker at a speed of 100 r/min for 15 min. Afterwards, fish flesh was removed by a centrifugation at 200g for 5 min, and the supernatant was collected and centrifuged at 10,000 g for 10 min to obtain cell pellets of bacteria. Bacterial cells were washed with 2 mL sterile physiological saline and collected after a centrifugation at the same condition above. Bacterial DNA was extracted as described by Liu et al. (2018a). Three parallel bacterial DNA samples collected from individual fillets were submitted to PCR amplification, respectively. The 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAA-3′) primers with different barcodes were used for PCR amplification of V3−V4 regions of 16S rRNA genes. PCR reactions were performed in triplicate in a final volume of 20 μL, containing 10 ng of template DNA, 0.4 μL of FastPfu Polymerase, 0.8 μL of each primer (5 μM), 2 μL of 2.5 mM dNTPs, 4 μL of 5 × FastPfu Buffer, and double distilled water. The resulting PCR products were detected and extracted by 2% agarose gels electrophoresis, and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.). Afterwards, quantification of purified PCR products was performed using QuantiFluor™-ST (Promega, U.S.), according to the manufacturer's instructions.

Composite amplicon samples including sub-samples from each triplicate was used in Illumina-sequencing. That is, purified amplicons were pooled in equimolar, and they were paired-end sequenced (2 × 300 bp) on an Illumina MiSeq platform, according to the standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP179819). Raw fastq files were demulti-plexed and quality-filtered by Trimmomatic and merged by FLASH using QIIME (version 1.17). Operational Taxonomic Units (OTUs) were clustered using UPARSE (version 7.1, http://drive5.com/uparse/) with a 97% similarity cutoff, and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by a RDP Classifier (http://rdp.cme.msu.edu/) against the silva (SSU115) 16S rRNA database using a confidence threshold of 70%. To minimize the difference of sequencing depth/read number across samples, an averaged OTU table generated at the minimum sample read number was used for further analysis. Alpha indices used to reflect the sequencing depth (Coverage), richness (ACE estimator and Chao1 estimator), and diversity (Shannon index) were determined by MOTHUR (version v.1.30.1, http://www.mothur.org/). A barplot of microbial communities (Fig. 2A) was generated using R programming language, and the clustered heat map (Fig. 2B) was generated based on Bray–Curtis distance using Vegan package in R.

2.10. Statistical analysis

Data were processed using Excel 2013 and expressed as mean ± standard deviation. Comparisons between different groups were made by the least significant difference (LSD) procedure using SPSS 22.0 software (SPSS Inc., Chicago, USA). For some data with uneven variance, rank sum tests with the Nemenyi method were performed.

3. Results and discussion

3.1. Extraction yield and total phenolics concentration of PPE

Light yellow extracts were obtained in our study, and the APPE was a little darker and browner than EPPE. The standard curve of gallic acid was $y = 2.42x + 0.0248$ ($R^2 = 0.9982$), where y is the absorbance value (Abs) and x is the concentration of standard gallic acid solution (mg/mL). Additionally, a proper addition of ethanol would produce a higher yield (Amyrgialaki at al., 2014). In fact, the extraction yield of APPE was 3.57 ± 0.30 g GAE/100 g fresh weight, and EPPE produced a higher yield of 3.89 ± 0.36 g GAE/100 g fresh weight.

3.2. Microbial analysis

3.2.1. Microbial enumeration

The initial total viable counts (TVC) of the control group, APPE group, and EPPE group were 3.14, 3.28, and 3.45 log CFU/g, respectively (Fig. 1). When compared with control, the TVC of treated samples increased more slowly during storage, and a reduction of more than 1 log CFU/g was observed on day 4 and day 5, which could be attributed to the antimicrobial property of PPE (i.e., including both APPE and EPPE). Meanwhile, there was no significant ($P > 0.05$) difference in TVC between the APPE and EPPE groups. Taking the 7 log CFU/g as an upper limit for freshwater fish (ICMSF, 1986), TVC of control and treated groups reached the limit (7 log CFU/g) on day 4–5 and day 6, respectively. Thus, the treated groups had a shelf-life of 1–2 days longer than the control, using TVC as a standard for judging shelf-life.

The spoilage microorganisms of chill-stored freshwater fish are generally dominated by psychrotrophic gram-negative bacteria such as *Pseudomonas*, *Aeromonas,* and *Shewanella* (the main H_2S-producing bacteria), all of which have extremely strong spoilage abilities (Gram, 1996; Liu et al., 2018b; Stohr et al., 2001; Wang et al., 2014). The bacterial counts for *Pseudomonas*, *Aeromonas,* and H_2S-producing bacteria were in a range of 2.0–3.0 log CFU/g on day 0 (Fig. 1), and they all

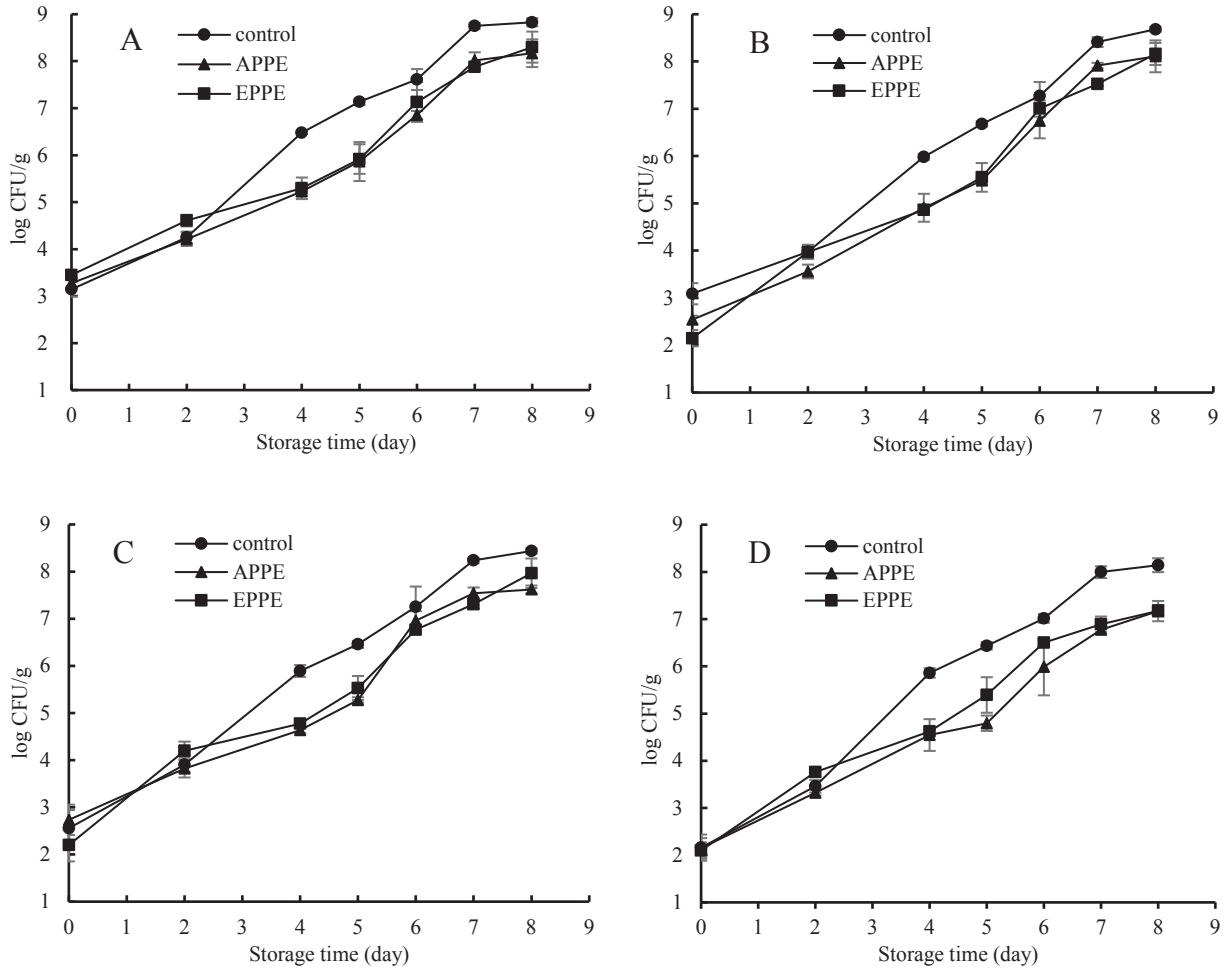

Fig. 1. Changes in TVC (A), *Pseudomonas* counts (B), *Aeromonas* counts (C) and H_2S-producing bacteria counts (D) of control and PPE treated samples during storage at 4 °C. (Control: untreated samples; APPE: samples treated with APPE solution; EPPE: samples treated with EPPE solution.)

underwent a similar increasing process as TVC. The PPE treatment decreased the counts of *Pseudomonas*, *Aeromonas*, and *Shewanella* significantly ($P < 0.05$) on day 4–5 and day 7–8, indicating that PPE was efficient in inhibiting the growth of *Pseudomonas*, *Aeromonas*, and *Shewanella*. However, counts of *Pseudomonas* and *Aeromonas* in control samples increased more quickly than that of H_2S-producing bacteria and reached nearly 9.0 log CFU/g at the end of storage, while the counts of H_2S-producing bacteria only reached 8.0 log CFU/g. This observation suggested that *Pseudomonas* and *Aeromonas* were more predominant in the late period of the spoilage process, which was consistent with the studies of Huang et al. (2017) and Wang et al. (2014). Moreover, the inhibitory effects of PPE on *Pseudomonas* and *Aeromonas* were similar, while PPE (especially APPE) showed a relatively better inhibitory effect on H_2S-producing bacteria. As a result, compared with control samples, a significant ($P < 0.05$) lower counts (1 log CFU/g) could also be observed in H_2S-producing bacteria even at the end of the experiment.

3.2.2. Microbiota composition in different samples

Microbiota composition of initial (day 0) and spoiled (day 8) fish fillets was analyzed through high-throughput sequencing based on Illumina MiSeq platform. In order to reveal the relation of microbiota change with sensory deterioration, samples on the day before sensory rejection point (day 5 for control and day 6 for treated samples) were used to analyze the microbiota composition of the middle-period of storage. After quality filtering, a total of 637,376 high-quality reads were obtained through the Illumina sequencing of bacterial 16S rRNA gene (Table 1). The coverage values of all samples were above 0.999, which indicated that the majority of bacterial phylotypes in samples were detected and recognized. Besides, the OTUs number, Shannon, ACE and Chao1 index of microbiota declined obviously during storage, which suggested the diversity and richness of microbial communities decreased with an increase of storage time.

Composition and relative abundance of microbiota at the genus-level among all samples were showed in Fig. 2A. *Photobacterium* was the predominant bacterial genus in the control group (49.6%), APPE group (42.5%), and EPPE group (63.4%) on day 0. The initial microbiota composition were kind of similar, while APPE and EPPE treatment decreased the relative abundance of *Acinetobacter* from 10.1% to 6.7%, and 1.5%, respectively. With an increase in storage time, microbial composition changed dramatically. *Acinetobacter*, *Pseudomonas,* and *Shewanella* (accounted for 65.8%, 23.1%, and 4.4%, respectively) became the most predominant bacteria in control samples on day 5. Given the results of microbial enumeration, PPE treatment decreased the TVC of *Pseudomonas* and *Aeromonas* on day 6. However, APPE and EPPE treatment had a more efficient effect on the *Acinetobacter* genus and reduced its relative abundance to 13.6% and 9.5%, respectively (Day 6). Thus, a corresponding increase occurred in the relative abundance of *Pseudomonas* and *Aeromonas*, which was because of a much more decrease in the sequencing number of *Acinetobacter* and other genera. At the end of storage, *Pseudomonas*, *Aeromonas,* and *Shewanella*, all of which have high spoilage potential according to previous studies (Gram, 1996; Liu et al., 2018b; Stohr et al., 2001; Wang et al., 2014), played an important role in sensory deterioration and became special

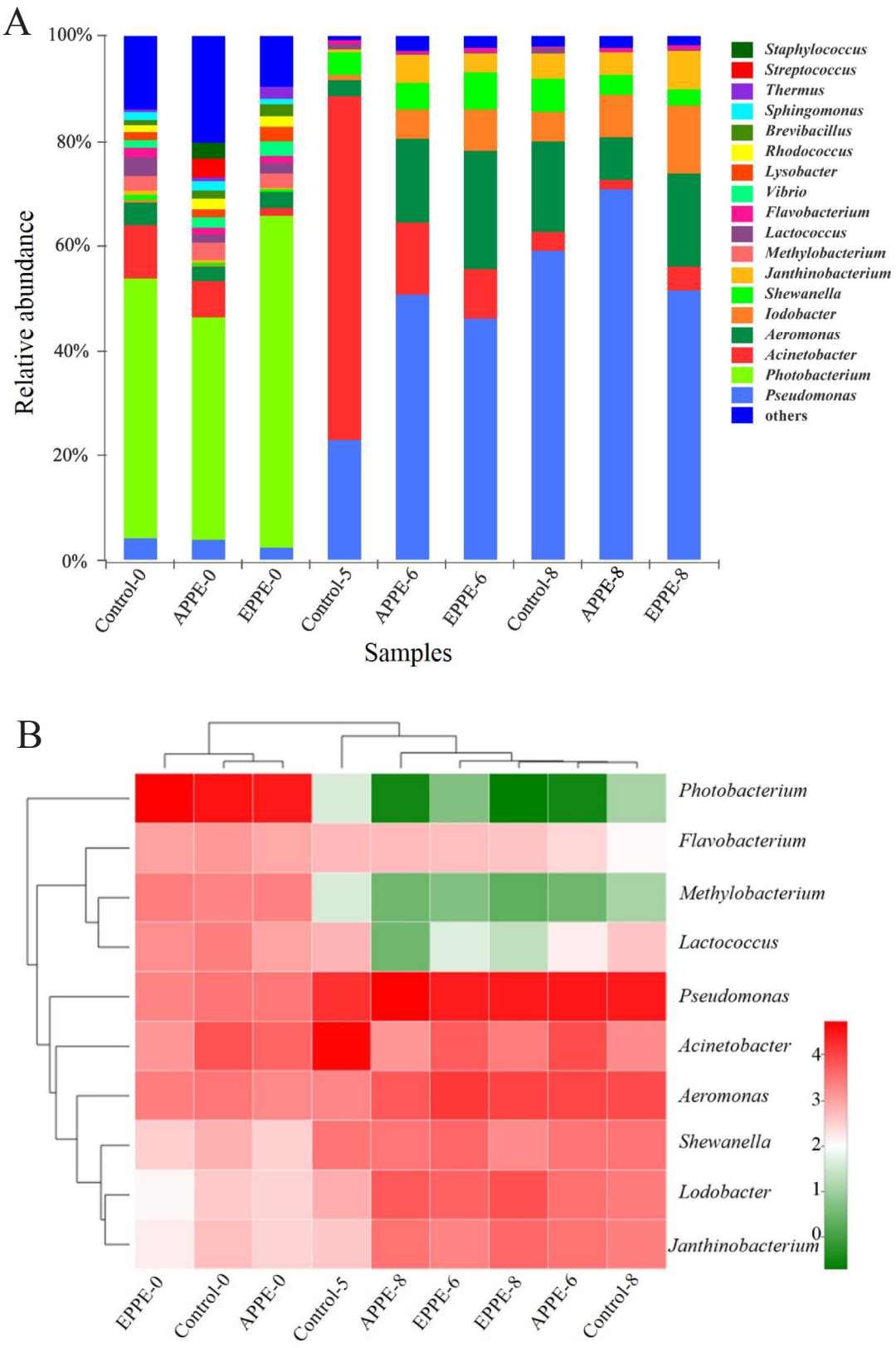

Fig. 2. Composition and relative abundance of microbiota at the genus level (A), community heatmap at the genus level (B). (Control-0: untreated samples at day 0; Control-5: untreated samples at day 5; Control-8: untreated samples at day 8; APPE-0: APPE treated samples at day 0; APPE-6: APPE treated samples at day 6; APPE-8: APPE treated samples at day 8 EPPE-0: EPPE treated samples at day 0; EPPE-6: EPPE treated samples at day 6; EPPE-8: EPPE treated samples at day 8.)

Table 1
Phylotype coverage and alpha diversity estimation of bacterial communities in bighead carp fillets during storage at 4 °C.

Samples	Read number	OTUs	Average length	Shannon	ACE	Chao1	Coverage
Control-day 0	71,468	234	447	2.73	239	240	0.9997
APPE-day 0	70,149	324	446	3.25	327	328	0.9998
EPPE-day 0	86,197	241	448	2.09	248	250	0.9996
Control-day 5	75,826	66	450	1.71	133	112	0.9995
APPE-day 6	63,008	64	450	1.99	102	81	0.9997
EPPE-day 6	63,433	57	450	2.04	66	62	0.9998
Control-day 8	61,657	50	450	1.91	70	65	0.9998
APPE-day 8	83,530	93	450	1.45	108	107	0.9997
EPPE-day 8	62,108	54	450	1.84	60	57	0.9999

Control: untreated samples; APPE: samples treated with APPE solution; EPPE: samples treated with EPPE solution. Day 0/5/6/8: storage time. OTUs: operational taxonomic units; Shannon: the Shannon index of community diversity; ACE: the ACE estimator of community richness; Chao1: the Chao1 estimator of community richness; Coverage: the good's community coverage.

spoilage microorganisms (SSOs). Huang et al. (2017) and Jia et al. (2018a) also identified these specific spoilage microorganisms in different freshwater fish species. Additionally, *Lodobacter* and *Janthinobacterium* also accounted for a large proportion of bacteria in spoiled fish and treated samples on day 6. However, spoilage potential and spoilage mechanisms of them need further investigation.

The notable difference in bacterial composition between control and treated groups on day 5 and day 6 revealed that PPE could influence the relative abundance of *Acinetobacter*, which is commonly the predominant bacterium in freshwater fish (Huang et al., 2018; Jia et al., 2018a; Liu et al., 2018a, 2018b). A similar effect on the relative abundance of *Acinetobacter* was also found using other plant-derived phenolic compounds, such as tea polyphenols (Jia et al., 2018b), which indicated that phenolic compounds might be a special inhibitor of *Acinetobacter*. Considering its remarkable predominance on day 5, *Acinetobacter* contributed significantly to the excess of TVC above the standard of 7.0 log CFU/g. Thus, an efficient inhibition of *Acinetobacter* was vital for a longer shelf-life of fish based on the TVC. Nevertheless, *Acinetobacter* was replaced rapidly by *Pseudomonas* and *Aeromonas* as storage time increased, which indicated that it might contribute less to the fish spoilage and sensory deterioration. Similar results were also observed by Liu et al. (2018b) and Wang et al. (2017). However, recent studies found that *Acinetobacter* could increase the spoilage potential of *Shewanella* through quorum sensing system and, thus, shorten the shelf-life of aquatic products (Zhu et al., 2018). Hence, the control of *Acinetobacter* is effective in retarding fish deterioration, though its spoilage ability is not as strong as some SSOs (e.g., *Pseudomonas*). Moreover, Zhu et al. (2018) found that *Acinetobacter* could boost the growth of *Shewanella*. This may partly explain why PPE had a more efficient inhibitory effect on *Shewanella* than on *Pseudomonas* and *Aeromonas*.

Because of the tremendous decrease in *Acinetobacter* in treated samples on day 6, the relative abundance of other bacterial genera like *Pseudomonas*, *Aeromonas*, *Lodobacter,* and *Janthinobacterium* increased. However, there was no significant increase in the relative abundance of *Shewanalla,* which indicated that PPE might have a stronger inhibitory effect on *Shewanella* than on the other two SSOs. This observation was consistent with the results from microbial enumeration. As a result, PPE slightly decreased the relative abundance of *Shewanella* from 6.38% (control group) to 3.99% (APPE group), and 3.03% (EPPE group) at the end of storage. Moreover, the EPPE group had more *Aeromonas,* but less *Pseudomonas* on day 6 and day 8, when compared with the APPE group. The phenomenon above suggested that components in APPE might have a better inhibitory effect on *Aeromonas* and that EPPE might be more effective at inhibiting *Pseudomonas,* which was not obvious in the microbial enumeration results.

A heat-map uses the type and depth of color to represent the relative abundances of different genera (Fig. 2B). Cluster trees of samples and the top 10 genera were constructed on the upper and left side of the figure, respectively, based on the similarity in abundance of genera. The clustering analyses of samples indicated that microbial composition of APPE samples on day 6 and day 8, and that of EPPE samples on day 6 and day 8, were all similar to that of control samples on day 8 (Fig. 2B). This was because PPE decreased the relative abundance of *Acinetobacter* so obviously that the microbial composition of treated groups on day 6 had already been close to that of samples at the end of storage. On the other hand, the inhibitory ability of PPE on *Pseudomonas* and *Aeromonas* was similar, so the PPE treatment failed to cause a significant change in microbial composition at the end of storage.

3.3. Color analysis

Color is an important factor that influences the acceptability of food products (Guerrero et al., 2015). Color values of fillets were characterized as L*, a*, and b* during chilled storage (Table 2). There was

Table 2
Color changes of control and PPE treated samples during storage at 4 °C.

Color indexes	Groups	Storage time (day)						
		0	2	4	5	6	7	8
L*	Control	44.37 ± 0.98^{Aa}	46.41 ± 2.39^{Ab}	45.08 ± 3.69^{Aab}	49.39 ± 0.58^{Bcd}	48.91 ± 3.06^{Bc}	49.69 ± 2.15^{Bc}	50.94 ± 2.47^{Bd}
	APPE	45.61 ± 1.76^{Aa}	46.47 ± 1.59^{Aa}	46.58 ± 1.85^{Aa}	45.57 ± 3.24^{Aa}	47.32 ± 1.21^{ABa}	45.47 ± 2.16^{Aa}	45.62 ± 2.22^{Aa}
	EPPE	45.23 ± 2.15^{Aa}	46.92 ± 1.49^{Aa}	45.48 ± 3.12^{Aa}	45.29 ± 2.70^{Aa}	44.51 ± 2.47^{Aa}	46.06 ± 2.43^{Aa}	47.44 ± 1.41^{ABa}
a*	Control	0.02 ± 0.22^{Ca}	-0.098 ± 0.55^{Ba}	0.09 ± 0.48^{Ba}	-0.58 ± 0.20^{Ab}	-0.78 ± 0.17^{Bbc}	-1.15 ± 0.24^{Ac}	-1.23 ± 0.46^{Ac}
	APPE	-1.16 ± 0.06^{Aa}	-1.07 ± 0.05^{Aa}	-0.86 ± 0.16^{Aa}	-1.00 ± 0.48^{Aa}	-1.15 ± 0.10^{Aa}	-1.01 ± 0.28^{Aba}	-1.02 ± 0.14^{Aa}
	EPPE	-0.61 ± 0.26^{Bab}	-0.42 ± 0.26^{Aba}	-0.50 ± 0.41^{Aab}	-0.56 ± 0.18^{Aab}	-0.54 ± 0.15^{Bab}	-0.67 ± 0.41^{Bab}	-0.95 ± 0.07^{Ab}
b*	Control	-1.21 ± 0.63^{Aa}	-1.14 ± 0.75^{Aa}	-0.90 ± 0.32^{Aa}	-0.68 ± 0.43^{Bb}	1.12 ± 0.61^{BCb}	1.03 ± 0.49^{ABb}	1.12 ± 0.37^{Ab}
	APPE	0.70 ± 0.24^{Ba}	0.70 ± 0.38^{Ba}	0.56 ± 0.50^{Ba}	0.75 ± 0.20^{Ba}	1.36 ± 0.15^{Cb}	1.43 ± 0.15^{Bb}	1.48 ± 0.12^{Ab}
	EPPE	-0.21 ± 0.32^{Ca}	-0.09 ± 0.23^{Ca}	0.08 ± 0.50^{Babc}	0.00 ± 0.35^{Aab}	0.37 ± 0.15^{Abc}	0.60 ± 0.33^{Acd}	1.05 ± 0.35^{Ad}

Note: Same superscript capital letters (A-C) in a column indicate no significant differences in the column ($P > 0.05$) and same superscript lowercase letters (a-d) in a row indicate no significant differences in the row ($P > 0.05$). (Control: untreated samples; APPE: samples treated with APPE solution; EPPE: samples treated with EPPE solution.)

no significant difference between the control and the two treated groups for initial L* values. However, the APPE treatment caused significant changes in initial values of both b* and a*, which were mainly due to the greenish and yellowish color of the APPE solution. This kind of negative effect on the initial color was relatively less for the EPPE group. Thus, EPPE-treated samples were more likely to be accepted by consumers. Similar results were also found in many other plant phenolic extracts, such as thinned young apple extract, grape seed extract, and clove bud extract (Berizi et al., 2016, 2018; Shi et al., 2014; Sun et al., 2017). Undesirable colors were derived mainly from some yellow-colored phenolic compounds like phlorizin and chlorogenic acid (Sun et al., 2017).

During storage, the L* value of the control group increased significantly ($P < 0.05$) from 44.37 (day 0) to 50.94 (day 8), and a significant ($P < 0.05$) difference in L* value between the control and treated groups appeared from day 5 (Table 2). The b* value of the control group increased as storage time progressed, but the a* value decreased. That is, the color of flesh became yellowish and greenish during storage, which was undesirable to consumers. When compared with the control, the b* value of the EPPE group increased more slowly and became significantly ($P < 0.05$) lower than that of control samples after day 5. Finally, the significant ($P < 0.05$) difference in b* value disappeared with a complete deterioration of all groups at the end of storage. Similar results were obtained also for the a* value of the EPPE group. Moreover, APPE also slowed the deterioration of color, although it caused significant changes in the initial a* and b* values. As reported, color changes during storage could be due to oxidation and degradation of proteins and lipids, which could change the absorption and scattering of light, and produce some oxidation products that affect color (Mørkøre, 2006; Shi et al., 2014; Sun et al., 2017). For example, the shift to a more yellowish hue is often associated with lipid oxidation (Guerrero et al., 2015; Shi et al., 2014). Therefore, the protective effect of PPE on flesh color may be attributed to its antioxidant activities that prevent oxidation of lipids or proteins. In addition, Liu et al. (2018b) found that *Pseudomonas helmanticensis* and *Aeromonas sobria*, both of which are dominant bacteria species in refrigerated bighead carp fillets, caused green and yellow discoloration of bighead carp flesh, respectively. This suggested that PPE's inhibitory effect on spoilage bacteria was likely to be another contributor to color protection.

3.4. Sensory evaluation

The initial odor and texture attribute scores of treated samples were 4.9 and 4.8 for the EPPE group, respectively, and 4.8 and 4.7 for the APPE group, respectively, which showed that the APPE and EPPE treatments did not change the initial odor and texture attributes (Fig. 3). However, the initial color attribute score and overall acceptability decreased slightly about 0.7 and 0.3, respectively, after EPPE treatment, as a result of the yellow color of the EPPE solution. Additionally, in our preliminary experiment, the color acceptable limit of APPE used in bighead carp fillets was 0.5 mg/mL, and both APPE and EPPE treatment at this concentration did not cause any unpleasant taste to bighead carp fillets. Thus, the concentration of 0.5 mg/mL was used in the experiment.

With increased storage time, all groups showed decreasing color, odor, texture, and overall acceptability scores, although scores of treated samples did not decrease as much as that of the control. For instance, the EPPE treatment prolonged the shelf-life of bighead carp fillets for about 2 days based on overall acceptability scores. As for color attribute only, the EPPE group retained better color attributes since the middle-period of storage, which correlated well with the results of color measurements. Additionally, the APPE treatment could have a similar performance in preventing odor and texture deterioration, but it didn't perform so well as EPPE in color scores and overall acceptability because of its heavier color. Hence, EPPE was a more suitable alternative for maintaining good sensory quality of freshwater fish fillets.

3.5. Chemical analysis

3.5.1. Changes in ATP-related compounds and K-value

Inosine 5′-monophosphate (IMP) is strongly associated with fish freshness (Huang et al., 2018). In the early storage period, the IMP concentration of all three groups increased sharply and reached the highest on day 4 (Fig. 4). As storage time increased, the IMP concentration of samples decreased. But the IMP concentration of treated groups (especially APPE group) decreased more slowly than that of control samples, which indicated that PPE inhibited the further degradation of IMP.

During the entire storage period, IMP was degraded to HxR and then converted to Hx under the action of both autolytic and bacterial enzymes (Hernández-Cázares et al., 2011). Hx has a bitter taste, and thus contributes to the off-flavor of fish products (Huang et al., 2018). Fortunately, treatment with PPE inhibited the decomposition of IMP and HxR and, thus, retarded the increase of Hx. As a result, the Hx concentration of treated groups was obviously lower than that of the control from day 5. Similar trends and results were also found in the HxR value. However, due to the rapid conversion from HxR to Hx, the rate of increase of HxR in the control group was much lower when compared with its Hx value. As a result, the difference in HxR value between the control and the treated groups was not so significant as that of the Hx value. This phenomenon suggested that PPE prevented the further degradation of HxR. Given the results of microorganism enumeration, the obvious effect of PPE in inhibiting the degradation of ATP-related compounds might be attributed to its inhibitory effect on spoilage bacteria. On the other hand, Cowan (1999) pointed out that phenolic compounds could inactivate bacterial enzymes by interacting with the sulfhydryl group of proteins. Therefore, PPE might inactivate extracellular enzymes like IMP phosphohydrolase and nucleoside phosphorylase, which catalyze the conversion of IMP to HxR, and HxR to Hx, respectively (Hong et al., 2017).

K-value is used widely to evaluate the freshness of fish. The initial K-value of the control group, APPE group, and EPPE group were 13.2, 12.4, and 14.1, respectively. With the degradation of ATP-related compounds in fish flesh, K-value increased among all groups. However, the K-value increased more slowly in treated groups than in the control group, due to the inhibiting effect of PPE on the degradation of ATP-related compounds. In addition, APPE seemed to be more effective in attenuating the degradation process of ATP-related compounds.

3.5.2. Changes in TVB-N and biogenic amines

The initial TVB-N of all three groups was in a low range of 7.84–8.87 mg N/100 g (Table 3). The TVB-N value of the control group fluctuated during the earlier period of storage (day 0–5) and increased significantly ($P < 0.05$) from day 6. Similar increasing trend was also observed in previous studies (Huang et al., 2017; Jia et al., 2018a; Zhang et al., 2017). The sharp increase of TVB-N usually occurs in the late period of storage, which may be because the generation of TVB-N requires a long degradation process to convert nitrogen-containing macromolecules to volatile small molecular compounds under the action of microbes. At the end of storage, the TVB-N value of the EPPE and APPE groups were 14.81 and 12.37 mg N/100 g, respectively, both of which were significantly lower than that of the control (19.74 mg N/100 g). This significant difference in TVB-N content could be attributed to the antimicrobial property of PPE. In addition, phenolic compounds in PPE could prevent protein decomposition caused by bacteria and hinder their capacity for oxidative deamination of nitrogenous compounds from non-protein sources (Fan et al., 2008; Saki et al., 2018). Liu et al. (2018b) found that *Shewanella* that was isolated from spoiled bighead carp flesh had a strong ability to produce TVB-N. As a result, the lower TVB-N content of the APPE group might be related to its better performance in inhibiting *Shewanella*, compared with the EPPE group.

Putrescine (PUT) and cadaverine (CAD) are formed from ornithine

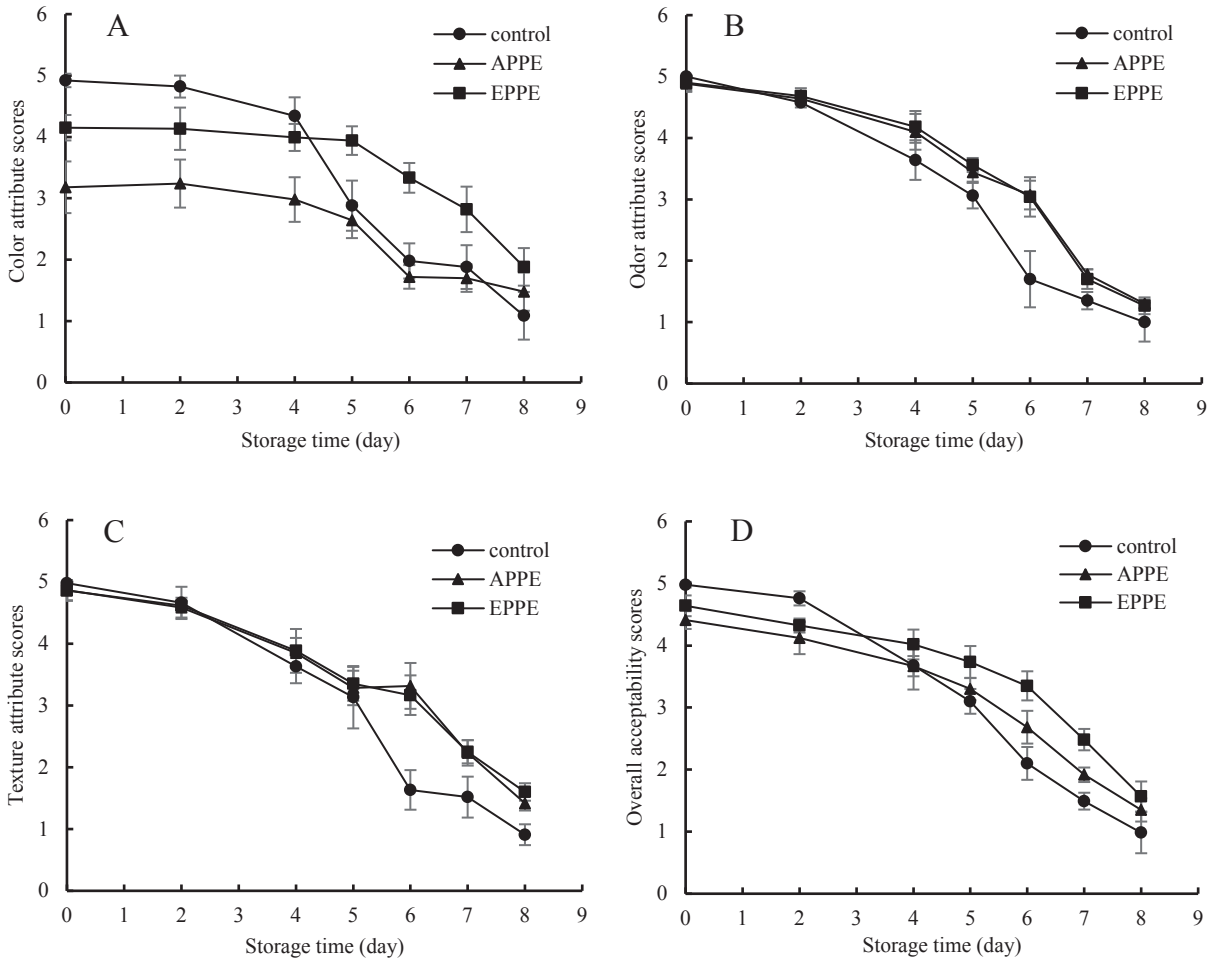

Fig. 3. Sensory evaluation of control and PPE treated bighead carp fillets during storage at 4 °C. (A: color attribute, B: odor attribute, C: texture attribute, D: overall acceptability. Sensory scores: 4.0–5.0 = good quality, 3.0–4.0 = acceptable quality, 2.0–3.0 = unacceptable quality, 1.0–2.0 = dislike extremely. Control: untreated samples; APPE: samples treated with APPE solution; EPPE: samples treated with EPPE solution.)

and lysine, respectively, mainly under the action of bacterial decarboxylase (Biji et al., 2016; Shi et al., 2012; Zhang et al., 2015). PUT and CAD are often used as the indicator of fish spoilage because they are well related to sensory deterioration of aquatic products (Chytiri et al., 2004; Shi et al., 2012; Zhang et al., 2015). In all three groups, PUT had initial concentrations < 1.0 mg/kg and maintained a low level for the next 4 days of storage. A sharp increase in PUT in the control group occurred on day 5, which was consistent with the time that TVC exceeded the limit of 7.0 log CFU/g. However, concentration of PUT in treated groups increased slowly and, thus, became significantly lower than that of the control from day 5. These results for PUT were consistent with odor scores in the sensory analysis, which indicated the preservative effects of PPE on samples. Meanwhile, there was no significant ($P > 0.05$) difference between APPE and EPPE groups, although the EPPE samples had a lower PUT concentration. Wang et al. (2017) reported that PUT could be produced under the action of *Pseudomonas*, *Aeromonas,* and *Shewanella*. Thus, the lower PUT concentrations in treated groups might be due to the bacterial inhibitory effect of PPE, based on microbial enumeration.

During storage, the concentration of CAD showed a trend similar to PUT. However, the sharp increase in CAD occurred later than PUT and, thus, the significant ($P < 0.05$) difference between the control and treated groups appeared on day 7. Thus, *Shewanella* may have greater CAD-producing ability than *Pseudomonas* and *Aeromonas*, because the changing trend in CAD was similar to that of *Shewanella* counts. This conclusion was also supported by Liu et al. (2018b), who confirmed that *Shewanella putrefaciens*, one of the most important spoilage bacteria in freshwater fish, was the primary producer of CAD in chill-stored bighead carp fillets.

4. Conclusion

The EPPE and APPE prepared in our study both exhibited a good preservative effect on chill-stored bighead carp fillets. Specifically, EPPE performed better relative to the index of color and biogenic amines inhibiting, but APPE was more effective in retarding the increase of TVB-N and K-value. Due to the unfavorable color of APPE, EPPE was preferred and prolonged shelf-life for about 2 days. Moreover, the microbial composition analysis indicated that *Acinetobacter* was the main bacteria in the middle-period of storage, but its dominant position was eventually replaced by SSOs (*Pseudomonas*, *Aeromonas,* and *Shewanella*). Meanwhile, microbiota composition was modified and changed by PPE (both APPE and EPPE). Nevertheless, the high-throughput sequencing results revealed that APPE had a better inhibitory effect on *Aeromonas*, but EPPE was better at inhibiting *Pseudomonas*. To conclude, PPE can be a potential preservative for chill-stored bighead carp fillets, although its unfavorable color needs to be modified and improved. In addition, further studies are recommended to elucidate the preservative effects of specific components in PPE and, especially, the mechanisms that inhibit spoilage bacteria like *Acinetobacter* and *Shewanella*.

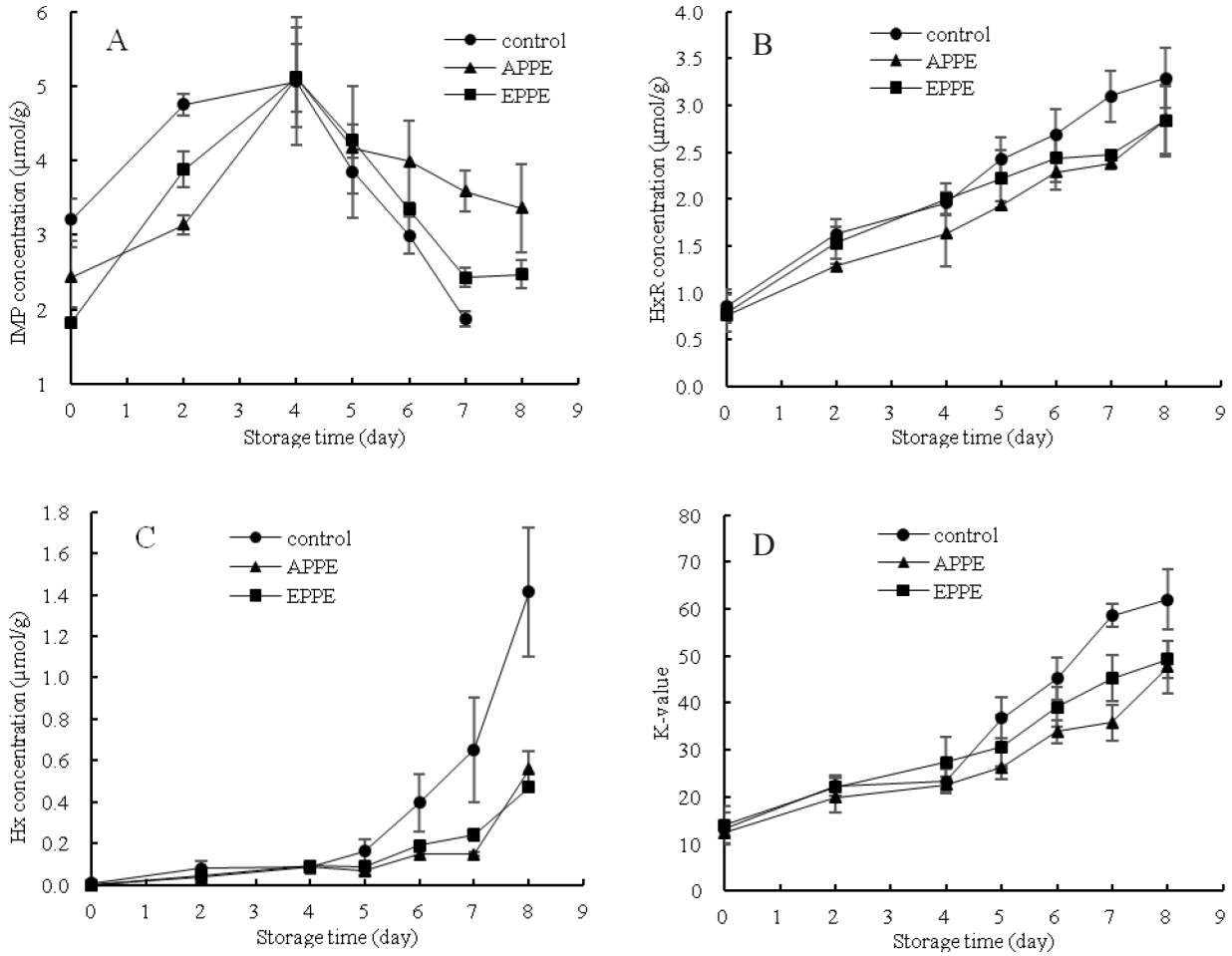

Fig. 4. Changes in IMP concentration (A), HxR concentration (B), Hx concentration (C) and K-value (D) of control and PPE treated bighead carp fillets during storage at 4 °C. (Control: untreated samples; APPE: samples treated with APPE solution; EPPE: samples treated with EPPE solution.)

Table 3
Changes in TVB-N and biogenic amines of control and PPE treated samples during storage at 4 °C.

	Groups	Storage time (day)						
		0	2	4	5	6	7	8
Putrescine (mg/kg)	Control	0.76 ± 0.16^{Ae}	0.46 ± 0.19^{Ae}	0.43 ± 0.06^{Ae}	2.07 ± 0.14^{Ad}	6.47 ± 1.20^{Ac}	17.39 ± 1.49^{Ab}	47.74 ± 31.73^{Aa}
	APPE	ND	ND	ND	1.84 ± 0.11^{Bd}	2.70 ± 0.01^{Bc}	5.96 ± 1.22^{Bb}	36.52 ± 0.75^{Aa}
	EPPE	0.41 ± 0.08^{Bd}	ND	ND	0.56 ± 0.27^{Bd}	2.13 ± 0.70^{Bc}	5.04 ± 2.53^{Bb}	40.57 ± 4.69^{Aa}
Cadaverine (mg/kg)	Control	3.62 ± 0.72^{Ab}	1.65 ± 0.14^{Ac}	1.80 ± 0.34^{Ac}	1.81 ± 0.79^{Ac}	2.10 ± 0.13^{Abc}	3.86 ± 0.91^{Ab}	10.48 ± 6.35^{a}
	APPE	2.23 ± 0.25^{Bb}	1.95 ± 0.14^{Ab}	1.83 ± 0.31^{Ab}	2.21 ± 0.40^{Ab}	1.82 ± 0.32^{Ab}	1.77 ± 0.17^{Bb}	9.67 ± 1.11^{a}
	EPPE	1.81 ± 0.36^{Bb}	1.88 ± 0.26^{Ab}	1.81 ± 0.33^{Ab}	1.93 ± 0.07^{Ab}	1.71 ± 0.23^{Ab}	2.04 ± 1.03^{ABb}	22.25 ± 3.85^{a}
TVB-N (mg N/100 g)	Control	7.84 ± 0.00^{Ae}	10.92 ± 0.00^{Acd}	10.92 ± 0.28^{Acd}	10.22 ± 0.40^{Ad}	11.62 ± 0.59^{Ac}	16.90 ± 1.44^{Ab}	19.74 ± 1.98^{Aa}
	APPE	8.40 ± 0.00^{Ac}	10.55 ± 0.81^{Aab}	11.06 ± 2.38^{Aab}	10.87 ± 1.32^{Aab}	10.17 ± 0.13^{Bb}	11.29 ± 1.91^{Bab}	12.37 ± 0.43^{Ba}
	EPPE	8.87 ± 1.59^{Ad}	10.45 ± 0.81^{Ab}	9.99 ± 1.33^{Ab}	10.87 ± 0.98^{Ab}	10.78 ± 0.59^{ABb}	11.48 ± 2.49^{Bb}	14.81 ± 2.04^{Ba}

Note: ND: not detected.
Same superscript capital letters (A-B) in a column indicate no significant differences in the column ($P > 0.05$) and same superscript lowercase letters (a-e) in a row indicate no significant differences in the row ($P > 0.05$). (Control: untreated samples; APPE: samples treated with APPE solution; EPPE: samples treated with EPPE solution.)

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