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The effects of CS@Fe_3O_4 nanoparticles combined with microwave or far infrared thawing on microbial diversity of red seabream (*Pagrus major*) fillets based on high-throughput sequencing

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ABSTRACT

The present study investigated the effects of CS@Fe_3O_4 nanoparticles combined with microwave or far infrared thawing on microbial diversity of red seabream (*Pagrus major*) fillets in terms of thawing loss, pH, TVB-N, classical microbiological enumeration and high-throughput sequencing, and the same parameters were also studied for 24 h after thawing. Four thawing methods were used: microwave thawing (MT), far-infrared thawing (FT), CS@Fe_3O_4 nanoparticles combined with microwave thawing (CMT) and CS@Fe_3O_4 nanoparticles combined with far-infrared thawing (CFT). The results showed that CFT and CMT had lower values of pH and TVB-N compared to the FT and MT. Based on conventional microbial count analysis, CFT and CMT samples also maintained lower TVC, pseudomonas and LAB counts. Using high-throughput sequencing analysis, Compared with FT and MT, CFT and CMT samples showed a significant decrease in the proportion of the *Pseudomonadaceae* flora. However, the proportion of *Pseudomonas, Bacillaceae* and *Thermaceae* also increased significantly after 24 h of storage, which indicated that become the predominant microbiota in red seabream (*Pagrus major*) fillets.

1. Introduction

Nowadays, the food industry and consumers usually use frozen storage as an important preservation method to keep seafoods fresh so that seafoods are frozen for a long periods. It is also important to adopt more efficient, convenient, economical and safer methods of thawing before processed (Ersoy et al., 2008). A common thawing method in food industry and consumer households is to use microwave to shorten the thawing time and increase the efficiency (Phinney et al., 2017; Cai et al., 2018; Ersoy et al., 2008). It satisfies the requirement of fast heating and effectively maintains the nutritional quality and flavor of food. Microwave thawing could promote the retention of ascorbic acid and anthocyanins in strawberries (Holzwarth et al., 2012). However, microwave thawing is not uniform and may lead to local overheating. Infrared radiation is an electromagnetic radiation that propagates and converts into heat in the form of waves (Cai et al., 2019; Llave et al., 2016). Generally, far infrared thawing is the absorption of far infrared energy by food through changes in the state of molecular vibration, and thawed by radiation heating. Far-infrared heating provides efficient heat to food, which does not require a heat transfer medium and can reduce the processing time but may also cause local overheating (Rastogi, 2015; Backi, 2018).

Red seabream (*Pagrus major*) is one of the most important species for commercial coastal fisheries in China, South Korea and Japan. It was selected due to its good taste, high nutritive value, fast growth, and rich physiological active substances (Perez-Enriquez et al., 1999; Fukunishi et al., 2013). However, the spoilage in aquatic products often occurs with the growth and metabolism of microorganisms and affects the safety of foods in the market. Therefore, frozen storage is a usual method to prevent spoilage and prolong the shelf life of foods. It is also

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important to take appropriate and effective thawing methods before industrial circulation or human consumption.

As a natural product, chitosan is nontoxic, biocompatible, easy modified properties with unique physiological activity (Li et al., 2015). When heated, magnetic nanoparticles (MNP) promotes heat conduction in the paramagnetic state, causing polar molecules to move violently. MNP combined with radio frequency, far infrared and microwave, had the advantage of shortening the thawing time and evenly distributing the temperature when heated have been confirmed by many studies (Wang et al., 2014; Cao et al., 2018). So, trace amounts of chitosan magnetic nanoparticles (CS@Fe_3O_4 nanoparticles) were used in this study.

High throughput sequencing technology known as “next generation sequencing (NGS)" technology, have widely been used in analyzing the microbial diversity and variations of microbial community because of their advantages of detecting both unculturable microorganisms and low abundance microorganisms (Besser et al., 2018; Reuter et al., 2015). Direct extraction of total DNA from test samples for analysis avoids the traditional method of microbial isolation and culture. (Parlapani et al., 2018; Fei et al., 2018). At present, the high-throughput sequencing technology has been used in many aquatic products such as Chinese traditional fermented fish (Zang et al., 2018), silver carp (*Hypophthalmichthys molitrix*) (Jia et al., 2018), and lightly salted bighead carp (*Aristichthys nobilis*) (Liu et al., 2017). So, the high-throughput sequencing technology combined with traditional microbial culture method could help us to accurately explore the dominant microbiota at the level genus and species.

Nanotechnology faces great challenges in improving the quality and safety of food in the food industry (Neethirajan and Jayas, 2011). Combining CS@Fe_3O_4 nanoparticles with microwave or far infrared thawing maybe compensate for the defect of microwave or far infrared technology. The aim of the present study was to investigate the microbial community composition of red seabream with different thawing methods by high-throughput sequencing and to analyze using classical microbial enumeration. In the meantime, the changes of chemistry indicators in all samples were also determined to evaluate the effects of different thawing methods on the red seabream fillets and the storage process after thawing.

2. Materials and methods

2.1. Reagents

CS@Fe_3O_4 nanoparticles were got from Xi'a ruixi Biological Technology Co., Ltd (Xi'an, China). All other chemicals were bought from Fengchuan Chemical Reagent Technologies Co., Ltd (Tianjin, China) and were analytical grade. The deionized water (200 L, Sanda Shui Beijing Technology Co., Ltd, Beijing, China) was used during all experimental work. All mediums were purchased from Hopebio Bio-Tech Co. (Qingdao, Shandong, China).

2.2. Sample preparation

A total of 9 fresh red seabreams with an average weight of 0.9–1.2 kg, were purchased from a local aquatic market (Jinzhou, China) and transferred to the laboratory alive within 20min. Subsequently, Fresh red seabreams were stunned with a stick and skinned and gutted, filleted from back and washed with sterile cold water. One group of fillets was taken as fresh sample (FS), the other fillets were frozen in a −20 °C freezer for 24 h and left to thaw. These fillets were prepared for four different treatments: Microwave thawing (MT), chitosan magnetic nanoparticles plus microwave thawing (CMT), far-infrared thawing (FT), chitosan magnetic nanoparticles plus far-infrared thawing (CFT). And then half of the fish fillets were stored in the refrigerator at 4 °C for 24 h to determine all the parameters again.

2.3. Thawing processing

2.3.1. Microwave thawing (MT)

A group of frozen fillets was put into the microwave (NN-DF382M, Panasonic Appliances Microwave Oven Co., Ltd., Shanghai, China) and the thawing process was performed at a power of 300 W and at a frequency of 2450 MHz. A temperature recorder sensor (Elitech RC-4, Jingchuang Electric Co., Ltd., Xuzhou, Jiangsu, China) was used to detect until its core temperature ended at 0 °C.

2.3.2. CS@Fe_3O_4 nanoparticles plus microwave thawing (CMT)

A set of frozen fillets was placed in a beaker and added with the thawing solution. The concentration of CS@Fe_3O_4 nanoparticles solution was 0.1 mg/mL. Make sure the fillets were completely immersed in CSMNP solution. After that, the beaker was put into the microwave oven. The thawing condition and thawing end point were performed as Section 2.3.1.

2.3.3. Far-infrared thawing (FT)

A tomographic ark (CX-1, Jiapeng Technology Co., Ltd, Shanghai, China) was used to thaw fish fillets, in which two infrared tubes (4–14 μm, 12 × 300 mm, 300 W, Yuanxiang Electric Appliance Co., Ltd, Lianyungang, China) were installed on both sides. Then, a group of fish fillets was brought into a 500 mL beaker in a tomographic ark at a temperature of 10 °C. The temperature process termination were performed as Section 2.3.1.

2.3.4. CS@Fe_3O_4 nanoparticles plus far-infrared thawing (CFT)

The thawing process of CFT was similar to that of FT. A group of frozen fillets was placed in a beaker and added with the thawing solution. To ensure the effect of CSMNP solution, the fillets were completely submerged in the solution. The concentration of CSMNP solution was 0.1 mg/mL. Then the beaker was placed into the tomographic ark at a temperature of 10 °C. The temperature process termination were performed as Section 2.3.1.

2.4. Thawing loss

Thawing loss was calculated using the following equation:

Thawing loss (%) = (M_1-M_2)/$M_1$where M_1 is the weight of frozen sample and M_2 is the weight of the thawed sample.

2.5. pH

Five grams of sample were homogenized with 50 mL of deionized water for 1 min using a homogenizer. A digital pH meter (FE20K, Mettler Toledo, Switzerland) was used and the measurement was performed in triplicate.

2.6. TVB-N

Total volatile bases nitrogen (TVB-N) was measured by automatic kjeldahl determination method. The 10 g sample was placed in a distillation tube and then added with 1 g of magnesium oxide. The automatic kjeldahl apparatus (Kjeltec 8400, FOSS, Denmark) was immediately connected and the samples were measured according to the set conditions in triplicate.

2.7. Microbiological analysis

10 g red seabream samples were taken aseptically and transferred to a stomacher bag, and 90 mL of 0.1% peptone water with salt (NaCl, 0.85%, w/v) were added. The mixture were homogenized using a flap homogenizer (BagMixer 400 W, Shanghai, China) for 3 min. 0.1 mL homogenate solution was spread on the agar plate (Yu et al., 2018) and decimal dilutions of homogenates were done for three times.

2.8. Samples preparation for DNA extraction

Total bacterial genomic DNA samples were extracted using the metagenomic. DNA was extracted from all samples using the TIANamp Marine Animals DNA Kit (Tiangen Biotech Co., Ltd, Beijing, China) according to the manufacturer's instructions. Nine DNA samples were extracted from the dorsal muscle of the same red seabream and stored at −20 °C prior to further analysis. The quantity and quality of extracted DNAs were measured using a nucleic acid protein detector (BioPhotometer Plus, Eppendorf, Germany) and agarose gel electrophoresis, respectively.

2.9. 16 S rDNA amplicon pyrosequencing

PCR amplification of the bacterial 16 S rRNA genes V4 region was performed using the forward primer 515 F (5' - GTGCCAGCMGCCGCGGTAA - 3') and the reverse primer 806 R (5' - GGACTACHVGGGTWTCTAAT - 3'). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR components contained 25 μl of Phusion High-Fidelity PCR Master Mix, 3 μl (10 μM) of each Forward and Reverse primer, 10 μl of DNA Template, and 6 μl of ddH_2O. Thermal cycling consisted of initial denaturation at 98 °C for 30 s, followed by 25 cycles consisting of denaturation at 98 °C for 15 s, annealing at 58 °C for 15 s, and extension at 72 °C for 15 s, with a final extension of 1 min at 72 °C. PCR amplicons were purified with Agencourt AMPure XP Beads (Beckman Coulter, Indianapolis, IN, USA) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2 × 150 bp sequencing was performed using the Illlumina HiSeq 4000 platform (Guhe Info Technology Co., Ltd, Hangzhou, China).

2.10. Statistical analysis

Sequence data analyses were mainly performed using QIIME and R packages (v3.2.0). OTU-level alpha diversity indices, such as Chao1 richness estimator, ACE metric (Abundance-based Coverage Estimator), PD_whole_tree, Shannon diversity index, and Simpson index, were calculated using the OTU table in QIIME. Biochemical and microbial counting analyses were done in triplicate. The data were shown as the mean ± standard deviation and analyzed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) for one-way analysis of variance and Duncan's multiple range test. The significant differences between means with varying storage times and groups were set at $P < 0.05$. BioProject accession number is PRJNA605708.

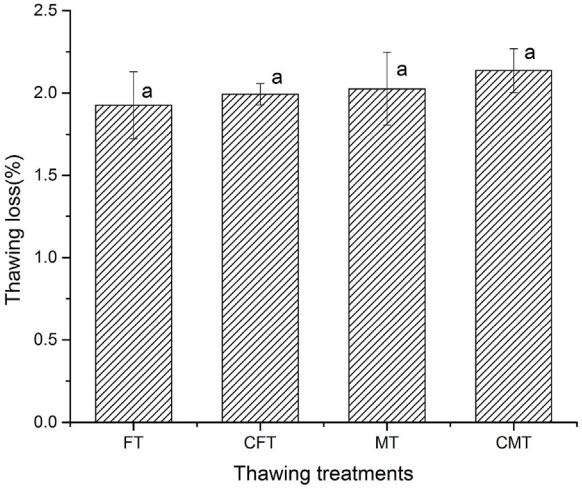
3. Results and discussion

3.1. Thawing loss

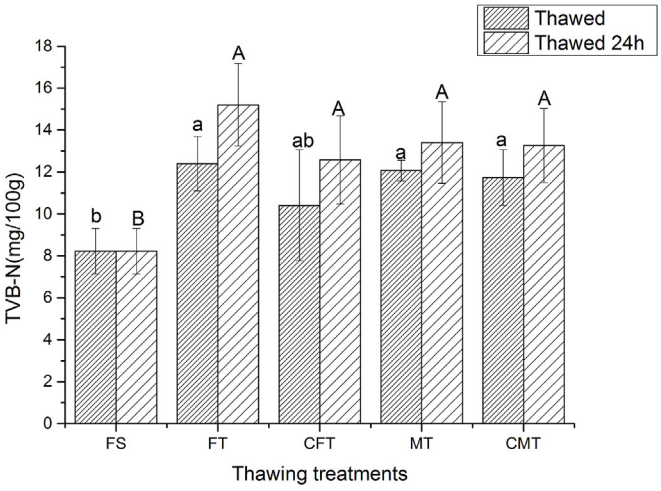
Thawing loss is an important index to measure the thawing quality of fish, which is related to the texture, water migration and tissue state of fish. When frozen fishes are thawed, the ice crystals in the body are melted and cause thawing loss. In this study, Fig. 1A showed that the thawing losses of MT and CMT were higher by 2.02% and 2.13% than the FT and CFT samples, respectively. Although there was no significant difference between the four groups and this indicated that the FT and CFT samples could better maintain the quality of the red seabream after thawing. The FT sample had the lowest thawing loss and higher thawing loss indicated poor meat quality (Li et al., 2017).

3.2. Changes in pH

Changes in pH values for all treated groups are shown in Table 1. Obviously, the pH values in all samples were decreased compared to the FS. The decrease of pH value might be closely related to anaerobic digestion of muscle glycogen or microbial and enzyme activities (Ozogul et al., 2016). The pH values in FT, CFT and CMT samples were significantly lower than the fresh sample. After 24 h of storage at 4 °C, changes in pH values for four groups were increased. Among them, the



(A)



(B)

Fig. 1. Effects of different thawing methods on thawing loss (A), TVB-N (B) in red seabream. Different capital or small letters represent significant differences ($P < 0.05$). Error bars show standard deviation.

Table 1
Effect of different thawing treatments on pH value, total viable counts, *Pseudomonas*, lactic acid bacteria lg (CFU/g) in red seabream muscle. (Values are mean ± standard deviation. "a–d" letters indicate significant differences ($p < 0.05$)).

	pH	TVC	*Pseudomonas*	LAB
FS	6.51 ± 0.020^{a}	3.59 ± 0.08^{a}	2.58 ± 0.06^{b}	3.00 ± 0.11^{b}
FT	6.19 ± 0.032^{b}	3.25 ± 0.06^{ab}	3.16 ± 0.11^{a}	3.59 ± 0.20^{a}
CFT	6.22 ± 0.010^{b}	3.10 ± 0.10^{b}	2.93 ± 0.22^{a}	3.49 ± 0.21^{a}
MT	6.31 ± 0.015^{ab}	3.39 ± 0.14^{a}	3.24 ± 0.23^{a}	3.56 ± 0.07^{a}
CMT	6.26 ± 0.001^{b}	3.29 ± 0.05^{ab}	2.80 ± 0.09^{ab}	3.58 ± 0.05^{a}
A1	6.31 ± 0.011^{a}	3.48 ± 0.20^{a}	3.32 ± 0.09^{a}	4.08 ± 0.09^{a}
B1	6.33 ± 0.015^{a}	3.39 ± 0.09^{a}	3.19 ± 0.27^{a}	3.78 ± 0.02^{a}
C1	6.50 ± 0.015^{a}	3.58 ± 0.05^{a}	3.49 ± 0.15^{a}	3.76 ± 0.14^{a}
D1	6.45 ± 0.005^{a}	3.50 ± 0.04^{a}	3.12 ± 0.10^{a}	3.88 ± 0.03^{a}

CFT and CMT samples had the lowest pH values, which indicated that these two methods can better maintain the meat quality.

3.3. TVB-N

As shown in Fig. 1B, the initial TVB-N content of fresh sample were 8.22 mg/100 g. The TVB-N value was significantly ($p < 0.05$) increased after thawing. Among the four thawing methods, the TVB-N value of CFT was the lowest. At the same time, the CFT and CMT groups have lower TVB-N values than the FT and MT groups, indicating that CS@Fe_3O_4 nanoparticles thawing had better effects on the freshness of the fish fillets. After 24 h of storage at 4 °C, the FT group had the highest TVB-N value and the four treatment groups had significantly higher TVB-N values than the fresh sample.

3.4. Microbiological analysis

The total number of colonies is one of the most important quality evaluation index of aquatic products, which can reflect the degree of deterioration of the samples (Roiha et al., 2018). The changes in bacterial communities (TVC, *Pseudomonas*, and LAB) of all samples are presented in Table 1. The FT, CFT and CMT samples had lower TVC ($P < 0.05$) compared to FS throughout the thawing period. The *Pseudomonas* and LAB counts in four treated samples were significantly ($P < 0.05$) higher compared to the fresh sample, and there were no significant differences between them. Although the change was obviously less, both CFT and CMT samples maintained the lowest bacterial counts, which showed that the nano thawed solution might play an important role during thawing.

Generally, more clearly growth was observed on all four thawed media when stored at 4 °C for 24 h after thawing. The TVC was gradually increased and there was no significant difference compared with the fresh sample. Simultaneously, the number of *Pseudomonas*, and LAB bacteria in all treated samples was also obviously increased and was significantly higher than the fresh sample. Additionally, the number of *Pseudomonas* and LAB in the CFT and CMT groups was lower than that in the FT and MT groups, and total number of bacteria was also lower.

3.5. Sequencing analysis

For 16 S rDNA analysis, in total, 107,580, 96,082, 119,538, 89,059 and 101,093 pyrosequencing tags were obtained for the FS, FT, CFT, MT and CMT samples, respectively. At the same time, clean tags were reduced to 61,180, 59,702, 110,527, 57,485 and 88,733, respectively (Table 2). The analyses of OUTs, Ace index and Chao1, Simpson diversity index and Coverage are shown in Table 2. The lowest OUTs value for FT sample was 322, CMT (24 h) OUTs value was close to 500 with fresh sample and 350–470 for other samples. The overall coverage for all the samples was ≥99.8%, which meant that this level of sequencing identified almost all the microbial phylotypes in the red seabream fillets. In addition, the FT and MT samples had higher values of Shannon index, compared to CFT and CMT samples, which implied that microbial communities of red seabream fillets had higher bacterial diversity.

Similarly, after 24 h of storage at 4 °C, the CFT and CMT samples also had lower Ace, Chao1 and Shannon index than the FT and MT samples, which indicated that community richness and microbial communities bacterial diversity of red seabream fillets were decreased.

3.6. Bacterial composition in red seabream fillets

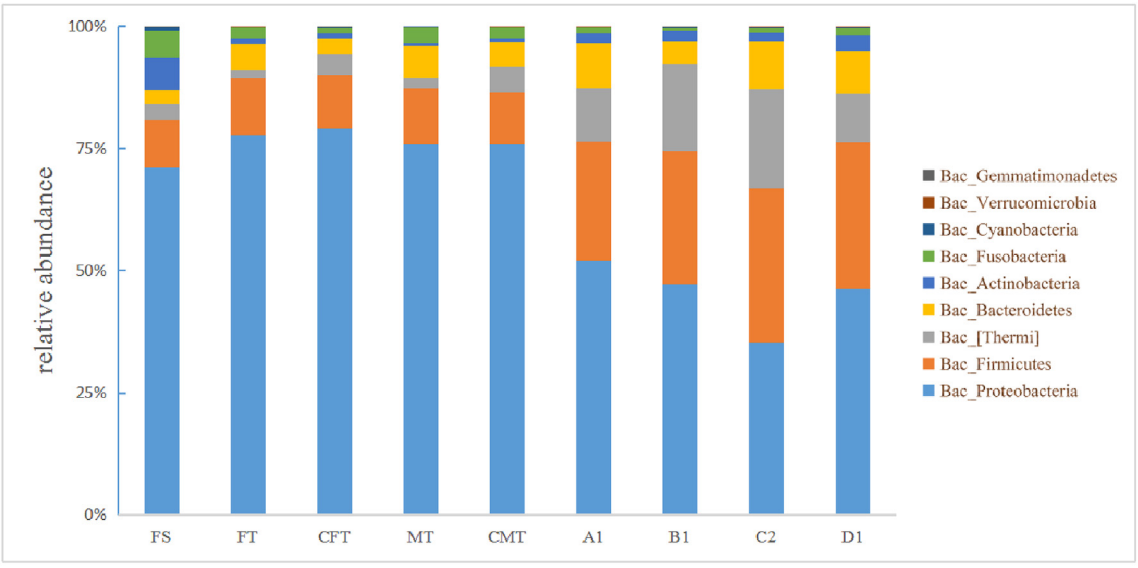
The composition and relative abundance of bacterial community in thawed fish fillets and during storage were analyzed by high-throughput sequencing. Percentage histograms were used to express the relative abundances of the top 10 phylum in all samples (Fig. 2A). *Firmicutes, Proteobacteria, Thermi* and *Bacteroidetes* were the major bacteria phyla in all samples. *Firmicutes, and Proteobacteria* were accounted for 9.5% and 70.0% in fresh sample, respectively. *Firmicutes* were increased from 9.5% to 11.4%, 11.0%, 12.2%, and 10.3% after four treatments. *Proteobacteria* were increased from 70.0% to 75.2%, 79.8%, 80.2% and 73.4%, respectively. Additionally, *Bacteroidetes* were also increased from 2.7% to 5.1%, 3.2%, 6.9% and 4.8%, respectively. With 24 h storage time, the dominance of the phylum *Firmicutes, Bacteroidetes and Thermi* were most apparent. In comparison, a significant increase in the bacterial families mentioned above was found in stored fillets. However, *Proteobacteria* were reduced to a minimum of 35.3% during the 24 h storage.

Furthermore, the results for major bacterial species in all samples at the family level are shown in Fig. 2B. *Vibrionaceae* and *Moraxellaceae* were the predominant species in fresh sample with composition proportions > 50%, followed by *Thermaceae*, *Pseudomonadaceae, Enterobacteriaceae* and *Bacteroidaceae. Vibrionaceae* were significantly increased after thawing. The percentage of major flora within *Pseudomonadaceae* in the CFT and CMT samples were decreased by 1.9% and 2.3% respectively, compared with FT and MT after the fillets were thawed. Obviously, *Vibrionaceae* were decreased rapidly during the 24 h storage, reached a minimum of 8%. The proportion of *Pseudomonas, Bacillaceae* and *Thermaceae* were also increased significantly, which indicated that these three microbial species might become the predominant species.

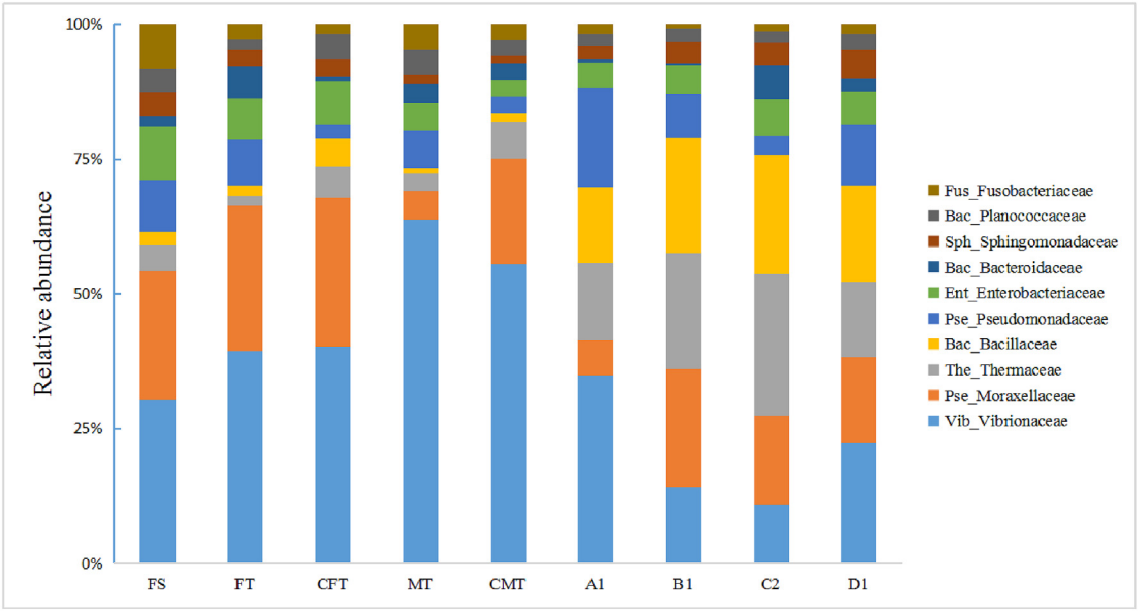
The samples were clustered according to the similarity between them and arranged in order. Then according to the similarity of the taxonomic units of different samples, the clustering results are arranged vertically. The heat-map was used to analyze and compare the microbiota differences and the dynamics and composition of bacterial communities in all samples (Zhang et al., 2017; Song et al., 2016; Li et al., 2018). The similarity between the samples and the community composition at the level of the genus can be seen from Fig. 3. Among the genus-level phylotypes, the color depth represented the relative abundance of the microbial genus. The abundance of bacteria in fresh sample was greater. Meanwhile, the bacterial composition of 24 h stored samples after thawing changed significantly compared with the fresh sample. Generally, the flora composition of the FT was different from that of the other three groups of samples, and the structure

Table 2
Comparison of alpha diversity estimation of the 16 S rRNA gene libraries by sequencing in red seabream fillets.

Treatments	Tags	Clean tags	Otus	Ace	Chao1	Shannon	Simpson	Coverage
FS	107,580	61,180	556	576.306	633.228	6.2621	0.9575	0.999
FT	96,082	59,702	322	384.878	433.892	5.1891	1.8542	0.999
CFT	119,538	110,527	376	423.260	426.892	4.5259	0.8240	0.998
MT	89,059	57,485	354	362.367	374.790	4.6597	0.8608	0.998
CMT	101,093	88,733	440	481.599	502.185	4.5335	0.82745	0.999
A1	105,125	99,179	470	509.055	523.929	4.2403	0.8578	0.999
B1	105,167	101,288	446	486.894	498.367	4.2054	0.9087	0.999
C1	87,453	62,053	387	504.802	571.182	5.5290	0.8721	0.999
D1	107,694	101,649	500	492.808	423.763	4.8945	0.9317	0.999



(A)



(B)

Fig. 2. Relative abundance at the phylum (A) and family (B) levels based on 16 s rRNA genes sequences of microbiota from red seabream fillets. (A1, B1, C1 and D1 were represented as FT, CFT, MT and CMT, which were stored in a 4 °C refrigerator for 24 h). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

similarity of the CFT and CMT flora was higher. The four groups of samples stored after thawing had higher similarity in structure.

4. Conclusions

High-throughput sequencing can provide detailed information on the species and abundance of red seabream microorganisms. The microwave or far infrared combined with CS@Fe_3O_4 nanoparticles thawing effectively delayed the increase of TVB-N and pH, while reducing the growth of microorganisms such as TVC, *Pseudomonas* and LAB. High-throughput sequencing analysis suggested that *Firmicutes, Proteobacteria, Thermi* and *Bacteroidetes* were the major bacteria phyla in the fresh sample. In the four groups of thawing treatment, the populations of *Pseudomonas* were lower in CFT and CMT groups. After 24 h of storage after thawing, *Pseudomonas, Bacillaceae* and *Thermaceae* were increased significantly and might become the dominant species. Further research is needed to better understand the active mechanism of CS@Fe_3O_4 nanoparticles combined with microwave or far infrared thawing and to apply them in practice to industrial process and consumer homes.

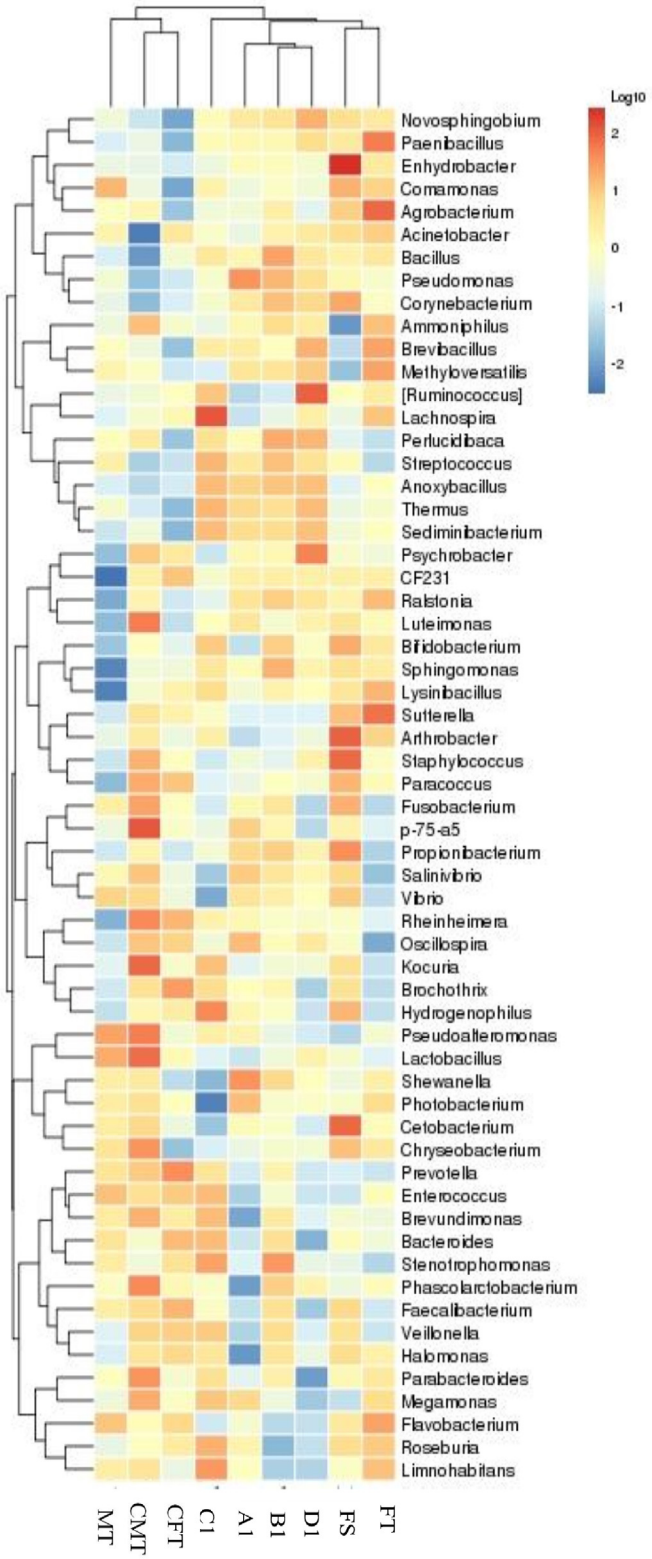


Fig. 3. Community heatmap analysis at the genus-level of different microbiota in red seabream fillets. (A1, B1, C1 and D1 were represented as FT, CFT, MT and CMT, which were stored in a 4 °C refrigerator for 24 h). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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