Acta Alimentaria 51 (2022) 1, 74–83
DOI: 10.1556/066.2021.00167

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Effects of various preservation treatments on diversity and abundance of microbial community in rice product (MiBa) during storage

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ORIGINAL RESEARCH PAPER

Received: July 16, 2021 • Accepted: October 20, 2021
Published online: February 9, 2022

ABSTRACT

To determine the most effective preservation method for MiBa (a traditional Chinese rice product), MiBa treated with 75% alcohol, 75% alcohol + inhibitor, ozone treatment; untreated (control); and raw rice were subjected to 16S rRNA gene and ITS three-generation sequencing by High-throughput Sequencing Technology. According to the results the preservation effects of different treatment methods ranked as follows: ozone treatment >75% alcohol treatment >75% alcohol+inhibitor > control. Bacterial composition analysis showed that the bacterial community on the surface of MiBa treated with ozone was dominated by genera *Leuconostoc* and *Serratia*. The fungal community consisted mainly of *Aspergillus* and *Alternaria*. In

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summary, ozone treatment proved to be the most effective in inhibiting microbial contamination during the storage of MiBa, effectively extending its shelf life.

KEYWORDS

MiBa, shelf life, high-throughput sequencing, diversity of microbial community

1. INTRODUCTION

Conventional physical preservation methods of rice products include low temperature storage (Shu et al., 2021), air control storage (Sasaki et al., 2007), electromagnetic field storage (Sung et al., 2007), biological agent storage (Hata et al., 2016), thermal sterilisation (Seiichiro et al., 2008), microwave sterilisation (Soni et al., 2020), and acid soaking (Liu et al., 2011). Commonly used preservatives include glyceryl monocaprylate, sodium diacetate, lysozyme, potassium sorbate, natamycin, and nisin (Rachtanapun et al., 2015). The preservatives presently used in rice products have common disadvantages such as high cost, unpleasant odour, and poor preservation ability (Skariyachan and Govindarajan, 2019). In addition, using a single preservative is undesirable, whereas more preservatives combined can effectively inhibit the growth and reproduction of spoilage microorganisms in rice products (Fu et al., 2003). As a traditional Chinese rice food with unique flavour, MiBa is popular with the consumers (Ji et al., 2007), but its shelf life limits MiBa commercialisation (Li et al., 2021). However, effective preservation method for MiBa has not yet been developed. Therefore, it is necessary to explore convenient, low-cost, efficient, and safe MiBa preservation methods to be able to expand the market of MiBa.

Microbial infection has always been the main cause of rice product spoilage and poses a threat to food safety of rice products. The study of microbial diversity of spoiled MiBa can reveal the main causes of spoilage and provide a certain research perspective and theoretical basis for prevention and control of MiBa spoilage. The study methods of microbial diversity have developed from conventional plate isolation and identification to polymerase chain reaction, and then to high-throughput sequencing (Reuter et al., 2015) and metagenomics (Ji et al., 2017). High-throughput sequencing is widely used nowadays, since it can sequence a large number of DNAs in a short time and analyse the complex microbial community structure rapidly. MiBa is one of the most important rice products in China, but its microbiological characteristics in storage have not been examined so far. There was one study on the microbiological characteristics of MiGao (a rice product similar to MiBa) during storage (Ji et al., 2007). Another study reported the lactic acid bacterium community composition and its dynamic change through the production process of rice noodles (another rice product similar to MiBa) (Li et al., 2015). The diversity of microbial community of rice noodles produced by different fermentation methods has also been reported (Geng et al., 2019). Few reports on the application of third-generation sequencing technology in the study of the microorganisms are available. Most research focused on fermented products such as fermented milk products (Mo et al., 2019).

In this study, high-throughput sequencing technology was used to study the microbial community diversity and characteristics of rice 408 (raw rice material of MiBa) and MiBa treated with several preservation methods. This study was aimed to extend MiBa shelf life and to improve the quality and safety of MiBa.

2. MATERIALS AND METHODS

2.1. Sampling and storage

Samples of MiBa were collected from a local village in Qingshui Township of Tengchong, Yunnan, China. All samples were collected and placed in sterile glass boxes, then immediately transported to the laboratory in an ice box. The samples were immersed respectively in a 1 L container filled with 75% alcohol (A1 Group) and 75% alcohol added with sterilisation agent containing 0.1 g nisin, 0.05 g natamycin, and 1 g potassium sorbate (A2 Group) for 10 s. The obtained two kinds of samples were drained quickly. Some raw samples were subjected to ozone disinfection (3G/H 70 W) treatment for 20 min (A3 Group) in a covered container. The MiBa without preservation treatment was set as the control group (CK1). The raw rice 408 soaked in 10 mL sterile water was set as another control group (CK2). All samples were vacuum-packed and stored at room temperature for 19 days.

2.2. DNA extraction and high-throughput sequencing

Total microbial community DNA was isolated from 0.25 g of MiBa per sample using the PowerSoil® DNA Isolation kit (*MO BIO Laboratories* Inc., Carlsbad, CA, United States) according to the manufacturer's instructions. Quality and concentration of the extracted DNA were checked by 0.8% agarose gel electrophoresis and ultraviolet spectrophotometry, respectively. High-quality DNA samples were amplified using primers targeting the full-length regions of the bacterial 16S rDNA and fungal ITS sequences. For bacterial community, the bacterial 16S rDNA were amplified using the universal bacterial primers 27F (5′-AGRGTTYGATYMTG GCTCAG-3′) and 1492R (5′-RGYTACCTTGTTACGACTT-3′). For the fungal community, ITS sequences were amplified using the primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4R (5′-TCCTCCGCTTATTGATATGC-3′). PCR amplification was performed in a total reaction volume of 20 μL, which contained 10 μL buffer, 0.4 μL DNA polymerase, 4 μL dNTP, 1 μL of each primer (10 μM), and 50 ng genomic DNA. The PCR was set up as follows: an initial denaturation step for 5 min at 95 °C, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min, and a final extension step at 72 °C for 7 min. After electrophoresis of the PCR products, they were purified, quantified, and homogenised to create a SMRTbell sequence library, and the marker gene was sequenced using the PacBio single molecule, real-time (SMRT) sequencing technology (Pacific Biosciences, Menlo Park, CA, United States). All the above-mentioned procedures were completed by Biomarker Technologies Corporation (Beijing, China)[1].

2.3. Data pre-processing and sequencing data bioinformatics analysis

Circular consensus sequencing (CCS) reads were obtained with the SMRT Link v8.0 after correcting the raw sub-reads. CCS reads were barcode-identified and length-filtered; the chimeras were removed thereafter using UCHIME v.8.1 (Edgar et al., 2011), and finally optimised-CCS reads were obtained. Optimised-CCS reads were clustered into operational taxonomic units (OTUs) at 97% similarity using the USEARCH v.10.0 software (Edgar, 2013), and the representative OTU sequences were annotated using the SILVA bacterial 16S rRNA gene sequence

[1]http://www.biomarker.com.cn/biocloud.

database (Release132)[2] (Quast et al., 2013) and the UNITE fungal ITS database (Release 8.1)[3] (Kõljalg et al., 2005) by a QIIME-based wrapper of RDP-classifier v.2.2 with a confidence cutoff of 0.8. The detected communities were identified and annotated at different taxonomic levels (phylum, class, order, family, genus, and species). Further analysis was done to calculate alpha diversity and richness of OTUs, and the community composition of each sample was determined at different classification levels. Finally, based on the above analysis, alpha diversity index was applied to analyse the microbial diversity, including the Chao1, Shannon, Simpson, and Ace indices, which were obtained with Mothur software (version 1.30.1)[4]. The Alpha diversity indices were calculated and displayed by the QIIME2[5] and R software (V3.1.1), respectively. Unweighted Pair-group Method with Arithmetic Mean (UPGMA) was used for analysis, hierarchical clustering of samples was carried out by Python language tools. Heatmap of samples was drawn using R language tool.

3. RESULTS AND DISCUSSION

3.1. Deterioration of MiBa in storage

The deterioration of MiBa with different treatments was different during storage. With the extended storage time, the surface of the MiBa began to change in colour and visible mould colonies were gradually forming. The vacuum-sealed MiBa was stored at room temperature for 4 days, after which the edge of CK1 began to have morphological changes and a small amount of mould forming could be observed (Fig. 1). With the extended time, the edges of group A1 and group A2 also began to have morphological changes, and a light growth of moulds was observed. Deterioration observations are presented in Table 1. Microorganisms decomposed sugars in MiBa to produce CO_2. With the extension of storage time, starch and protein in MiBa were decomposed by microorganisms to produce acid (Lu et al., 2010). During the storage period, the MiBa in all groups except A3 began to generate gas, and water generation was also observed in the MiBa with A1 and A2 treatments. The fresh-keeping effect of different treatments was ranked in the following order: ozone treatment (18 d) > 75% alcohol treatment (10 d) > 75% alcohol + inhibitor (7 d) > control (4 d), namely, A3>A1>A2>CK1.

3.2. Alpha diversity analysis

The Alpha diversity index values of each sample are shown in Table 2. According to ACE Index and Chao Index, the bacterium data indicated that CK1 exhibited the highest bacterial abundance, followed by A1 (38.98), A2 (36.07), A3 (31.03), CK2 (20.50) (ACE) and A2 (36.00), A1 (30.00), A3 (29.33), CK2 (20.50) (Chao). According to Shannon Index and Simpson Index, CK1 had the highest bacterial community diversity, followed by A2 (1.98), A3 (1.91), A1 (1.52), CK2 (1.41) (Shannon) and A3 (0.20), A2 (0.24), A1 (0.30), CK2 (0.39) (Simpson). Similarly, the fungus data showed that A2 group displayed the highest abundance, followed by A3 (18.03), CK2 (17.82), CK1 (17.25), A1 (13.00) (ACE) and CK1 (17.00), A3 (16.00), A1 (10.75), CK2

[2]http://www.arb-silva.de.

[3]http://unite.ut.ee/index.php.

[4]http://www.mothur.org/.

[5]https://qiime2.org/.

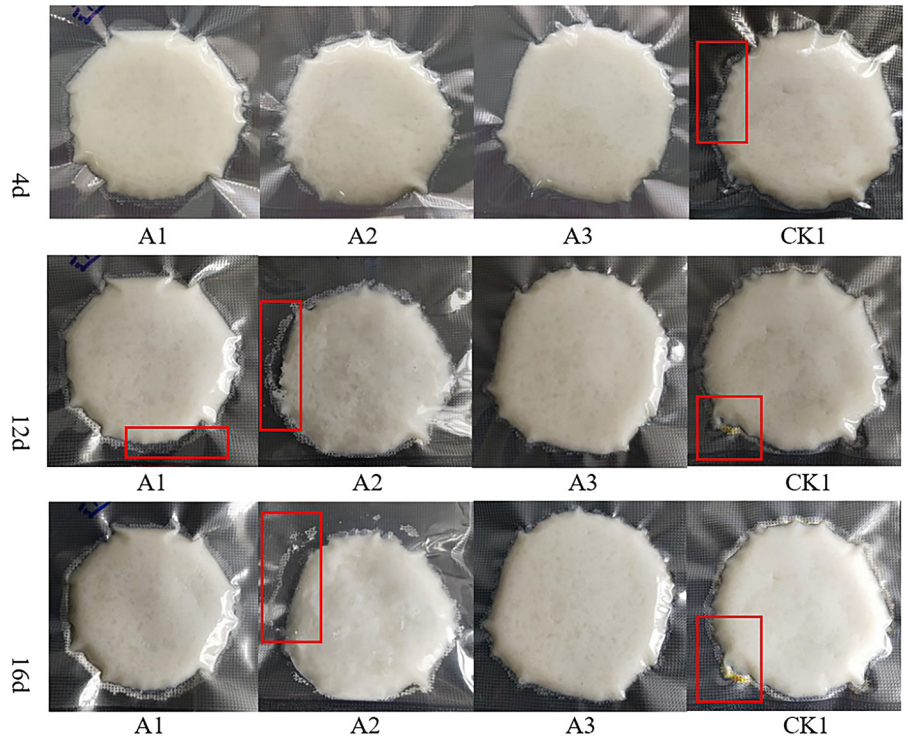

Fig. 1. Mildew changes during MiBa storage
A1, A2, A3, and CK1 represent different treatments, and different storage time is marked on the left

Table 1. MiBa deterioration after different preservation treatments

Samples	Preservation treatment	Start to mildew/d	Gas generation	Water generation
A1	75% alcohol	10	yes	yes
A2	75% alcohol + inhibitor	7	yes	yes
A3	ozone	18	no	no
CK1	control	4	yes	no

(9.60) (Chao). CK1 exhibited the highest fungal community diversity, A1 and A2 had relatively high fungal community diversity, A3 and CK2 displayed relatively low one with CK2 exhibiting the lowest. In summary, after the ozone treatment, MiBa in A3 group exhibited the lowest bacterial abundance (31.03 ACE, 29.33 Chao), but it displayed a high bacterial diversity (1.91 Shannon, 0.20 Simpson), and A3 group exhibited a relatively high fungal abundance (18.03 ACE, 16.00 Chao), but the lowest fungal diversity (0.15 Shannon, 0.95 Simpson).

Table 2. Alpha diversity index table (A-bacteria, B-fungi)

Group	Preservation treatment	Shannon	Simpson	ACE	Chao
A-A1	75% alcohol	1.52	0.30	38.98	30.00
A-A2	75% alcohol + inhibitor	1.98	0.24	36.07	36.00
A-A3	ozone	1.91	0.20	31.03	29.33
A-CK1	control	2.50	0.12	41.15	41.50
A-CK2	Raw rice	1.41	0.39	22.12	20.50
B-A1	75% alcohol	0.53	0.69	13.00	10.75
B-A2	75% alcohol + inhibitor	0.77	0.71	23.19	21.60
B-A3	ozone	0.15	0.95	18.03	16.00
B-CK1	control	1.07	0.54	17.25	17.00
B-CK2	Raw rice	0.02	0.99	17.82	9.60

3.3. Abundance clustering heatmap of microorganisms at genus level

The top 20 genera with high abundance were selected from five groups to draw the community composition heatmap (Fig. 2). Significant differences in community composition of microorganisms were observed among all five groups. According to community abundance similarity, bacterial communities in the five groups were clustered into four major categories (Fig. 2A). Category I consisted of three genera: *Gluconobacter* (A1 (12.39%) > CK1 (0.10%), those that were not listed were 0.00%), *Leuconostoc* (A1 (69.73%) > A2 (62.32%) > A3 (32.77%) > CK1 (11.77%) > CK2 (1.57%)), and *Weissella* (A1 (11.97%) > A2 (9.44%) > A3 (0.16%)). A1 and A2 groups exhibited high relative abundance of these three genera, of which *Gluconobacter* had a higher abundance in A1 group than in other groups (69.73%). Category II consisted of *Rosenbergiella* and *Staphylococcus*. *Rosenbergiella* had a certain abundance in A2 (0.65%) and A3 (0.62%), whereas *Staphylococcus* displayed the highest abundance in A2 (7.51%). Category III included five genera as shown in Fig. 2. All these five genera exhibited a relatively low abundance in samples except for CK2. Category IV included *Pantoea, Klebsiella, Serratia, Acinetobacter, Pseudomonas, Erwinia, Stenotrophomonas, Shewanella, Aeromonas,* and *Vibrio*. These bacterial genera except for *Pantoe* and*, Acinetobacter* displayed a more moderate relative abundance in A3 (13.29%, 18.11%, 8.34%, 1.03%, 0.02%, 0.00%, 0.00%, 0.00%) than CK1 (14.82%, 24.12%, 10.74%, 2.53%, 2.84%, 4.35%, 0.82%, 3.74%) groups. The relative abundances of these bacterial genera were extremely low in other groups (CK2, A1, A2).

As shown in Fig. 2B, there were significant differences in the fungal composition at the genus level among all groups. Fungal communities in the three treatment and two control groups were clustered into 4 major categories. These 8 genera in Category I had the highest abundance in the A2 group (alcohol+inhibitor). Among them, *Meyerozyma* and *Candida* had a certain abundance in CK1, *Meyerozyma, Udeniomyces, Debaryomyces,* and *Rhodotorula* had a certain abundance in A1. Category II consisted of *Mortierella* and *Wickerhamomyces*. *Mortierella* was more abundant in A1 (0.04%) than A2 (0.02%). *Wickerhamomyces* had a higher abundance in CK2 (99.71%). Category III consisted of *Aspergillus* and *Peniophora*, these two genera had the highest abundance in A3 (98.90%, 0.10%). All eight genera in Category IV had the highest abundance in CK1 with significant differences in the relative abundances of the fungal genera among various treatment groups, which might be due to the different surface environment caused by different MiBa preservation treatments.

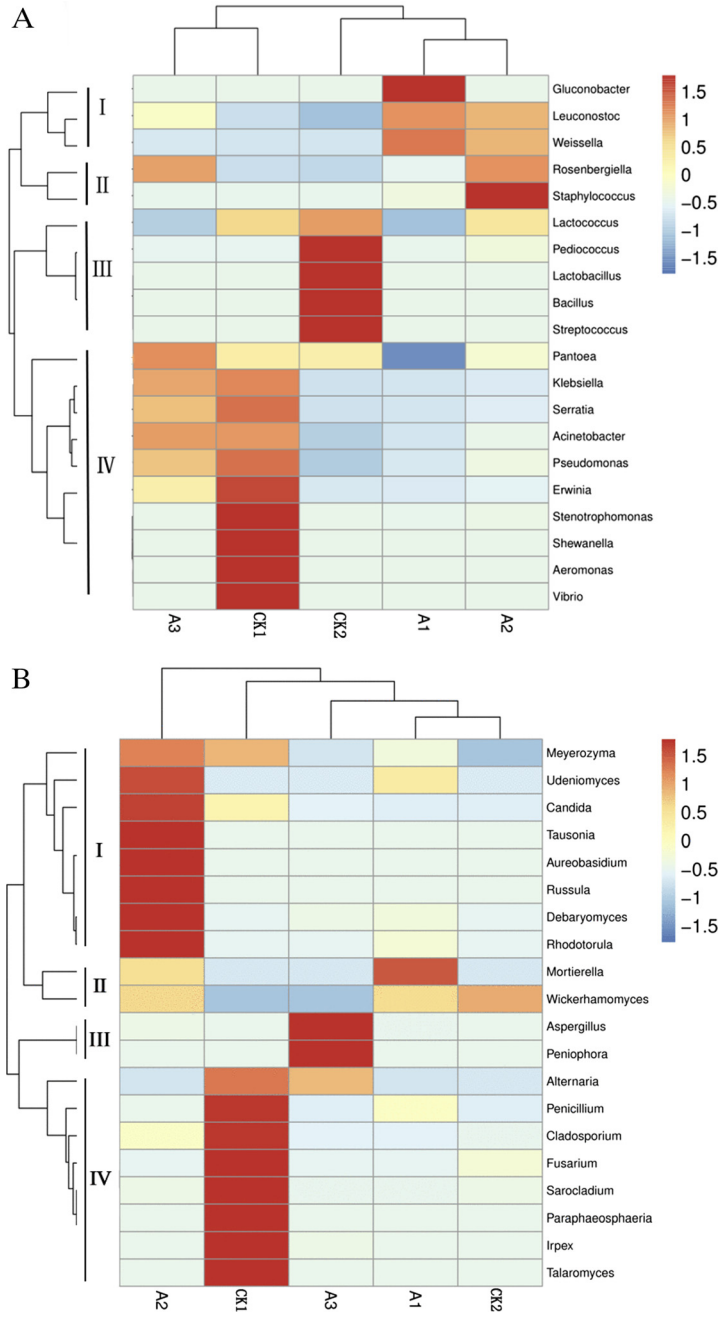

Fig. 2. Clustering heatmap at genus level (A-bacteria, B-fungi)
The horizontal clustering is the sample information. The vertical clustering is the genus information. The left clustering tree represents the genus clustering. The upper clustering tree indicates the sample clustering. The middle is the heat map

From the abundance clustering heatmap of microorganisms at genus level, it can be seen that some genera had higher abundance in A1, A2 than CK1. A1 and A2 exhibited significantly different microorganisms. The possible reason for such a difference lay in the different microbial environments on the surfaces of MiBas caused by alcohol treatment with (A2) or without (A1) antibacterial agent. The abundance decreased due to the ozone treatment in CK1. This might be due to the difference in preservation principles between alcohol and ozone treatment. Alcohol reduces or kills microorganisms by denaturing protein to cause irreversible damage and by destroying bacterial cell wall by lysing. Ozone sterilisation oxidised the enzymes decomposing the internal glucose in bacteria, or destroyed the cell organelles, DNA, and RNA of bacteria, thus resulting in the destruction of bacterium metabolism, lipoproteins of cell outer membrane, and lipopolysaccharides of cell inner membrane (Brodowska et al., 2017).

4. CONCLUSIONS

This study used high-throughput sequencing method to analyse the microbial community changes in four different preservation treatment groups and raw rice during the storage period of MiBa. The results showed that the preservation effects of different treatment methods ranked as follows: ozone treatment >75% alcohol treatment >75% alcohol+inhibitor > control. After ozone treatment (A3), the mould growth on MiBa was delayed.

Future studies will focus on the inhibition of the growth and reproduction of these specific spoilage microorganisms deteriorating MiBa by using combined treatments. As all rice products are made in a similar way, this study provides theoretical basis and practical reference for rice products storage.

ACKNOWLEDGMENTS

The present research was supported by the Yunnan Li Puwang Expert Workstation (202005AF50007), Cultivation of Agricultural Product Processing Team (202002AE320007-03), Green Food Brand Construction (Intensive Processing) of Fiscal Budget Major Special Project in Yunnan Province of China. Great gratitude goes to linguistics professor Ping Liu (Huazhong Agricultural University, Wuhan, China) for her work at English editing and language polishing.

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