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Dynamic changes of microbial flora in the pickled bergamot (*Citrus medica* L. *var. sarcodactylis*) - LaoXiangHuang (LXH) during aging

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Abstract

LaoXiangHuang (LXH) is a medical pickled fruit made of fresh finger citron (*Citrus medica L.* var. *sarcodactylis*) in Chaozhou, China. Aging is a process to strengthen the efficacy of Laoxianghuang (LXH), which is related to the activity of microorganisms. In this study, the changes of the microbial community of LXH within the three-years aging process were analyzed by high-throughput sequencing (HTS). According to alpha diversity results, bacterial abundance and diversity were higher than those of fungi. A total of 3 phyla and 13 bacterial genera were identified whereas 2 phyla and 4 fungal genera were identified. Meta-taxonomic of the overall bacterial communities in samples revealed that *Halomonas* spp. was the most abundant genus in all samples, which showed the first decresed, and then increased and decreased finally. The followed by *Nitriliuptor* spp., *Nesterenkonia* spp., *Weissella* spp., *Notinibacter* spp., *Streptococcus* spp., *Nocardiopsis* spp., *Acinetobacter* spp., *Ralstonia* spp., *Lactobacillus* spp. and unassigned genus. The unassigned genera (through the whole three-years fermentation period), *Ustilago* spp., *Malassezia* spp., *Aspergillus* spp. and *Schizophyllum* spp. were the predominant fungal genera. The result of PCOA and T-test analysis exhibited that the samples with different aging times have significant differences and are mainly reflected in *Halomonas*, unassigned bacterial genera, *Nitriliruptor*, *Nesterenkonia*, *Weissella*, *Intestinibacter*, *Streptococcus* and *Nocardiopsis*, *Lactobacillus, Acinetobacter, Ralstonia,* unassigned fungal genera, *Ustilago* spp. *Malassezia* spp. *Aspergillus* spp. and *Schizophyllum* spp., which may have important potential in promoting the aging process of LXH. This study helps us to understand the dynamic change of microorganisms of LXH during the aging process and provides a research basis for shortening the aging time of LXH in the future.

Keywords: finger citrus; *Citrus medica* L. *var. sarcodactylis*; microbial flora; aging.

Practical Application: The study showed the dynamic change of pickled bergamot (LXH) during aging. The results showed that the bacteria maybe play an important role in aging of LXH, which provide a basis for shorting aging time.

1 Introduction

Bergamot (*Citrus medica L.* var. *sarcodactylis*) originated from India and is now widely grown in the United States, Italy, France, Southeast Asia and other regions. It is used as both medicine and food, and is widely called finger citrus because of its shape of fingers (Gao et al., 2019b). Bergamot is mainly produced in Guangdong, Fujian, etc., and popular in Chaozhou, China. It contains a variety of beneficial compounds, including polysaccharides, flavonoids, cumarins, phenols, and carotenoids (Tsiokanos et al., 2021). Those compounds are used to remove phlegm, reduce coughing, relieve hypertension, and cure trachitis (He et al., 2014; Ma et al., 2021). However, the bergamot contains numerous bitter substances (Gupta et al., 2021). Thus, it is difficult to consume fresh fructus directly. The fructus with pungent odour should be processed before use to avoid exaggerated functional effects, reduce side effects, and modify energy properties (Wang et al., 2015). The processing is similar to that of Chinese herbal medicine. In the Chaoshan area, the fresh bergamot was salted, washed, dried, steamed, and immersed in Chinese medicine juice, and then aged in cylinders to form LaoXiangHuang (LXH) (Chen et al., 2020). During the aging time, the time longer, the colour deeper and functional efficacy better. The salting processing helps to penetrate actives into the kidney and improve curative effect (Weng et al., 2014). Processing crude medicine with herbal juices also plays an important role in the Chinese herbal medicine process. Honey can modify lung effects and improve anti-microbe effects (Monteiro, 2008). It also helps to prevent fatigue, and improve the spleen as well as thymus index in mice (Li et al., 2020).

Aging is also an important processing. According to previous studies, processed fingered citron tend to be more effective (Luo et al., 2020). Chen Xiaoai et al. used GC-MS, GC-IMS and electronic nose to analyze the volatile substances of LXH per 2 months within a year, and found that the volatile substances changed greatly after six months of fermentation, and new volatile flavor substances were produced (Chen et al., 2020). According to Jing He and Liu Sujuan et al. found that the quality of Chen Pi similar to LXH is related to the activity of microbes during aging (Liu et al., 2017; He et al., 2019).

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Wang fu et al. found that the fungus on the surface of Chen Pi could transform flavonoids and enhance the efficacy of Chen Pi (Wang et al., 2015). Zhong Junjie et al. successfully accelerated the aging process of Chen Pi by inoculating *Aspergillus niger* (Zhong et al., 2019). Thus, the microorganism is a major factor in the process of aging. However, the microbial changes of LXH during the aging process remain unknown. We hope to find out the microbial community that promotes aging in LXH by studying the evolution of its microbial colony and shortening the aging cycle of LXH.

Recently, high-throughput sequencing has been widely used to study the diversity of food microorganisms, e.g., kimchi, soybean and sufu (Xu et al., 2020; Kim et al., 2021; Zhao et al., 2021). In contrast to culture-based microbial assays, high-throughput sequencing provides a comprehensive analysis of both culturable and non-culturable microorganisms (Guo et al., 2020).

The changes of microorganisms during the aging process of LXH have not been detected. In this study, the composition and dynamic changes of LXH at different aging times were examined to identify the microbes that may improve the health attributes of LXH. Microbes may prove to be a valuable tool in the process of aging LXH.

2 Material and method

2.1 The manufacture of samples

The samples used in this study were provided by Zhancui food co, Ltd (Chaozhou, China). They were processed as follows: 1) Fresh finger citrus fruits were selected, washed, and pickled in saturated brine (concentration at above 36%) for six months. 2) The pickled fingers citrus was washed in running water for 12h to reach 3% saltness. 3) The treated finger citrus was immersed in Chinese medicine soup made up of equal amounts (accounting for 3% of raw materials) of liquorice, clove, cortex cinnamomic, foeniculum vulgare, and 28 °Bx sugar for a month. 4) The immersed finger citrus was dried at 50 °C for 24 hours. 5) The products were stored in a cylinder and manufactured as a batch per six months from early 2018 to later 2020. Five fruits were randomly collected from each batch, chopped and mixed as a parallel sample, and repeated three times as three parallel samples. It was then transported in dry ice to the lab and refrigerated under -18 °C for analysis. According to the aging time, the samples were named CT, 1Y-1, 1Y-2, 2Y-1, 2Y-2, 3Y-1, 3Y-2, respectively. The sample CT refers to the initial sample without aging. 1Y-1 refers to the sample manufactured in the later of 2020, whose aging time reached 6 months. 1Y-2 refers to the sample manufactured in the early of 2020, whose aging time reached 1 year. 2Y-1 refers to the sample manufactured in the later of 2019, whose aging time reached 1.5 years. 2Y-2 refers to the sample manufactured in the early of 2019, whose aging time reached 2 years. 3Y-1 refers to the sample manufactured in the later of 2020, whose aging time reached 2.5 years. 3Y-2 refers to the sample manufactured in the early of 2019, whose aging time reached 3 years.

2.2 DNA abstract and PCR amplification, Illumina MiSeq sequence

The total genic DNA was extracted using the HiPure soil DNA Kits B (Magen, Guangzhou, China) according to the manufacturer's instructions. The quality of DNA was investigated using 1% agarose gel electrophoresis and NanoDrop One. The oligonucleotide primer sequences " CCTACGGRRBGCASCAGKVRVGAAT" and " GGACTACNVGGGTWTCTAATCC" were amplified the V3 and V4 region, and "GTGAATCATCGARTC" and "TCCTCCGCTTATTGAT" were amplified the ITS2 region. The PCR amplification was processed with 25 uL mixture containing 2.5 uL TransStart Buffer, 2 uL dNTPs, 1 uL primer, 20 ng DNA polymerase, and according to the following procedure: degenerated at 94 °C for 5 mins, cycled 25 times with degenerated at 94 °C for 30 s, annealed at 57 °C for 30 s, 65 °C for 30 s, and was finally extended at 72 °C for 5 mins. The PCR products was detected through 1.5% agarose gel electrophoresis and purified using magnetic beads (AgencourtAMPure XP). The DNA library was built and accessed using Qubit 3.0 Fluorometer. Illumina MiSeq sequencer PE250 (Illumina, San Diego, CA, USA) were used to pair-end reads of 300 bp whereas MiSeq control software (MCS) was used for analysis.

2.3 Data analysis

The raw sequences were filtrated to get rid of the bar-codes, primers, short sequences (length < 200 bp), and low-quality bases (mass < 20). Filtering was also performed to reduce mistake due to the low-quality sequence. The sequences were then detected using UCHIME algorithm and annotated based on RDP Gold database. The sequences were finally referred to as the effective sequences after they were got rid of chimeric sequences for downstream statistical analysis. The effective sequences were clustered at 97% similarity of sequence and classified as the operation units (OTUs). The OPLS-DA were analysed using SIMCA 14.1 software (Umetrics, Sweden).

2.4 Nucleotide sequence accession numbers

The raw microbial sequences were uploaded to the NCBI sequence read archive (SRA) database with accession number of SRR15616637-SRR15616672.

3 Result

3.1 Alpha diversity

The alpha diversity of LXH is shown as presented in Figure 1 and Table S1. The alpha diversity index is used to determine the abundance and diversity of microbes in each sample. The good's coverage was greater than 0.98, which indicates that the sequencing depth was sufficient to reflect microbial status. Further, it was found that the bacterial abundances in the samples were significantly greater than that of fungi. On the other hand, the bacterial diversity of the samples also exhibits a greater diversity than fungi's besides 2Y-2. Although the indices of reads, Chao 1 and richness reveals the abundance of samples without significant change during aging time, the values tend to initially increased, then decreased and increased finally.

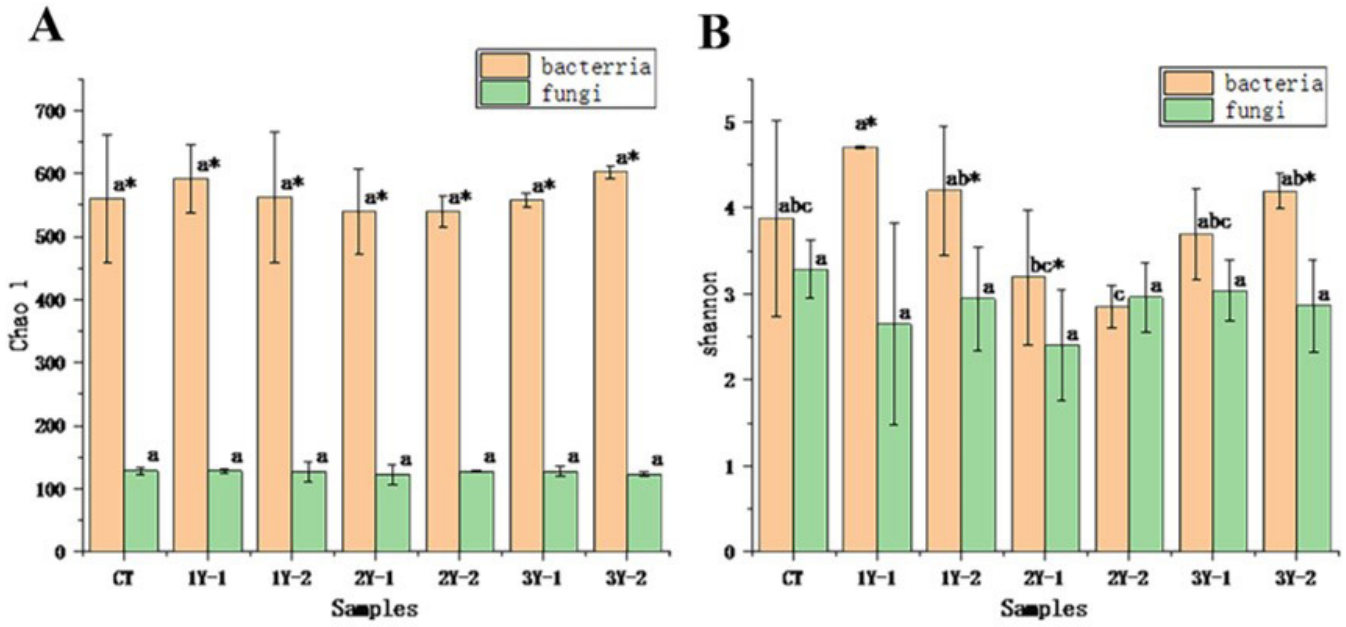

Figure 1. Alpha diversity indices in Chaozhou LXH during aging. The code refers to the collected sample time, e.g., 1Y-1 refer to the sample collected in the early of first year and 1Y-2 refer to the sample collected in the later of first year. Other samples were named as well. a, b, c, d, e. Statistical analysis was performed using one-way ANOVA (Duncan's test, Tukey's test, $P < 0.05$) and indicated that the significant difference between samples in difference aging times. The * indicated that the significantly difference between bacterial samples and fungal samples.

The indices of Shannon and Simpson showed that bacterial diversity significantly tend to initially increased, then decreased and increased finally. However, there are no significant change in fungal diversity and abundance during aging time.

Rarefaction curves were explored to show the adequate depth of the sequencing. It was found that the curve flattened when the rarefaction percentage reached above 70% (Figure 2). Compared with that of fungi, bacterial diversity was higher and this was similar to the result of the alpha diversity analysis.

3.2 Microbial diversity analysis of LXH during aging

The results of microbial composition showed that a total of 3 bacterial and 2 fungal phyla were obtained in LXH during aging. In addition, Proteobacteria (25.03~59.70%), Firmicutes (18.41~50.01%) and Actinobacteria (12.21~36.36%) were the main bacterial phyla (Figure 3A), while Ascomycetes (21.21~68.50%), Basidiomycota (2.80~22.01%), and unassigned phyla (28.70~70.59%) predominated (Figure 4A).

At the genus level (Figure 3B), *Halomonas* spp. (4.42~54.80%), *Nitriliuptor* spp. (4.57~18.83%), *Nesterenkonia* spp. (3.68~15.60%), *Weissella* spp. (3.24~12.09%), *Intestinibacter* spp. (3.19~9.23%), *Streptococcus* spp. (2.77~7.85%), *Nocardiopsis* spp. (0.79~4.55%), *Acinetobacter* spp. (0.57~5.88%), *Ralstonia* spp. (0.43~5.27%), *Lactobacillus* spp. (1.67~4.54%) and unassigned genus (9.70~22.44%) were the main genera, whose abundance was great than 1%. As shown in Figure 3C, the *Nitriliuptor* spp., *Acinetobacter* spp. and *Nesterenkonia* spp. had a downward trend thought the aging time. *Streptococcus* spp., *Intestinibacter* spp., *Weissella* spp., unassigned genus and *Lactobacillus* spp. significantly elevated in the first year and third year, and then declined in the second year. *Halomonas* spp., *Nocardiopsis* spp., *Stenotrophomonas* spp. and *Pseudomonas* spp. were enriched in the early or later of the second year. Results (Figure 4B) showed that the unassigned genera (77.82~97.98%) were the main genera through the aging time. More than 20% of *Ustilago* spp. was obtained at the beginning of the second year. Besides, there were *Aspergillus* spp. (0.15~9.71%), *Ustilago* spp. (0.13~1.63%), *Schizophyllum* spp. (0.09~9.66%) and *Malassezia* spp. (0.52~9.35%). According to the heatmap (Figure 3C), the fungal colonies were an alternation every 6 months. In the initial sample (CT), *Aspergillus* spp. was the predominance, while unassigned genus was the predominance in the first year. *Ustilago* spp. takes a predominant position in the early second year, while *Schizophyllum* spp. and *Malassezia* spp. take a predominant position in the early third year.

3.3 Analysis of the difference between LXH during aging

PCOA analysis of microorganism in LXH during aging time

In Figure 5A, in the principal co-ordinates analysis (PCOA) of bacterial communities during the aging time, the contribution rate of the first principal component is 68.8%, the contribution rate of the second principal component is 20.2%, and the cumulative contribution rate is 90.0%. The data indicated that the two principal components can include the main original data. The samples with similar aging time partially overlapped, while the samples with longer aging time were farther apart, which indicated that the aging time affect the bacterial colony of samples Figure 5B shows the PCOA analysis of fungal communities in

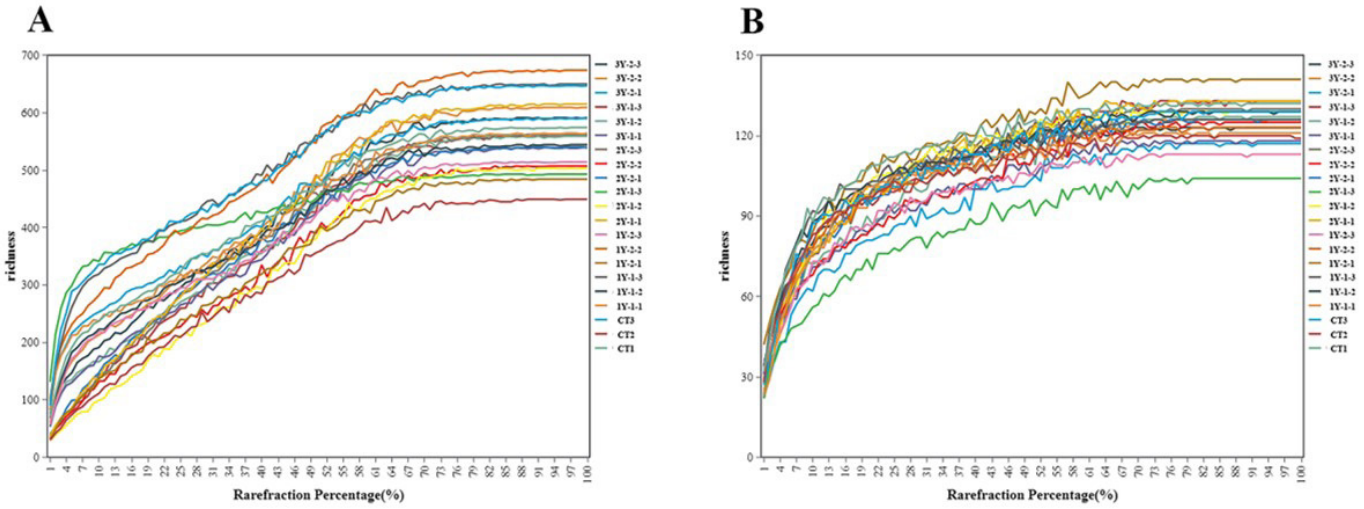

Figure 2. Rarefaction curves of LXH during aging. The code refers to the collected sample time, e.g., 1Y-1 refer to the sample collected in the early of first year and 1Y-2 refer to the sample collected in the later of first year. The last number 1, 2, 3 refer to the parallel sample. Other samples were named as well. The abscissa is the rarefaction percentage and the ordinate is the richness. Every line with different colour indicated a different sample.

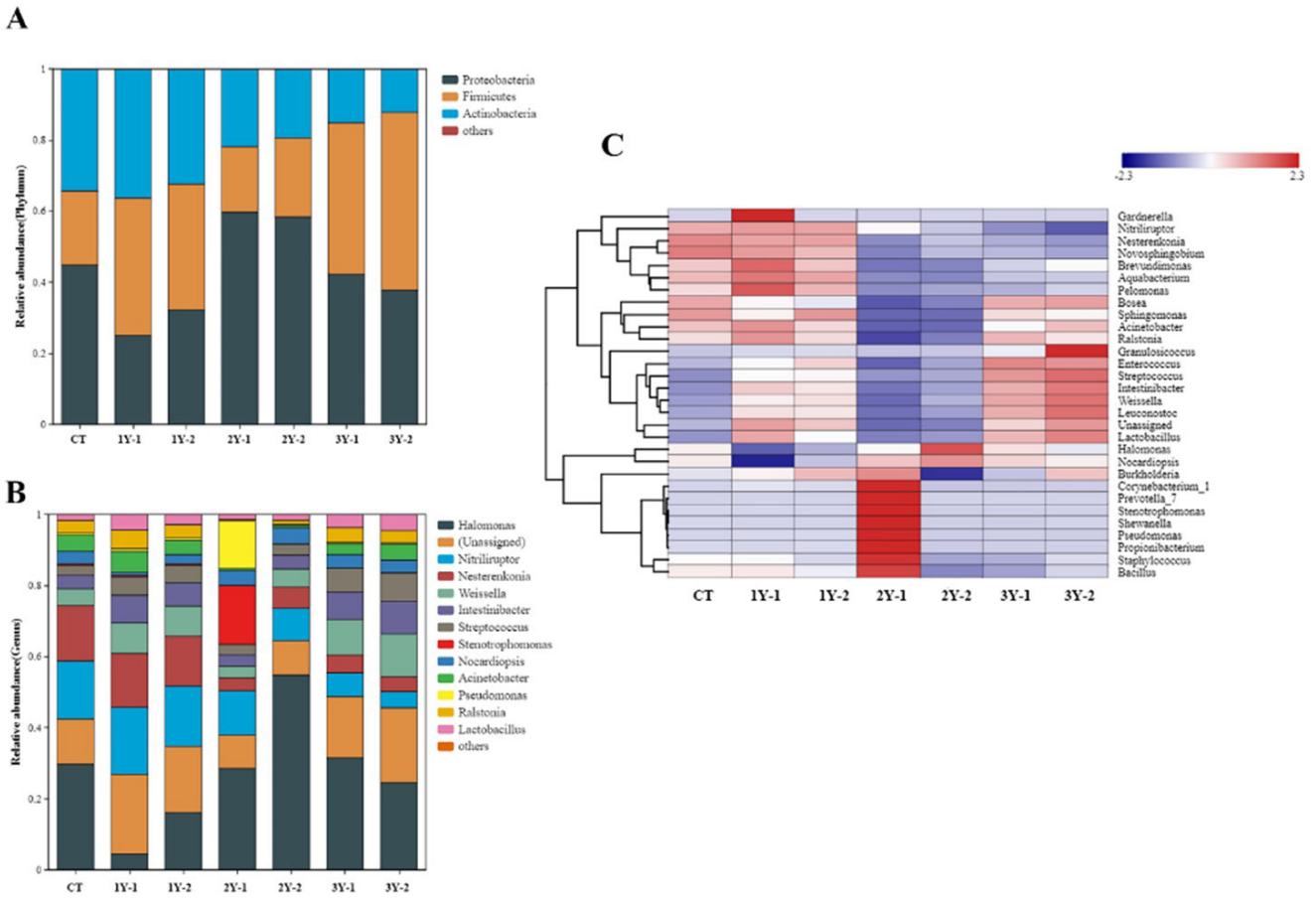

Figure 3. Bacterial composition of LXH at phyla level (A) and genera level (B), and the cluster heatmap (C) during aging. The code refers to the collected sample time, e.g., 1Y-1 refer to the sample collected in the early of first year and 1Y-2 refer to the sample collected in the later of first year. Other samples were named as well. The abscissa is the sample name and the ordinate is the relative abundance of difference bacteria. Every column indicated a group different sample and every column contained different percent bacteria with different colour.

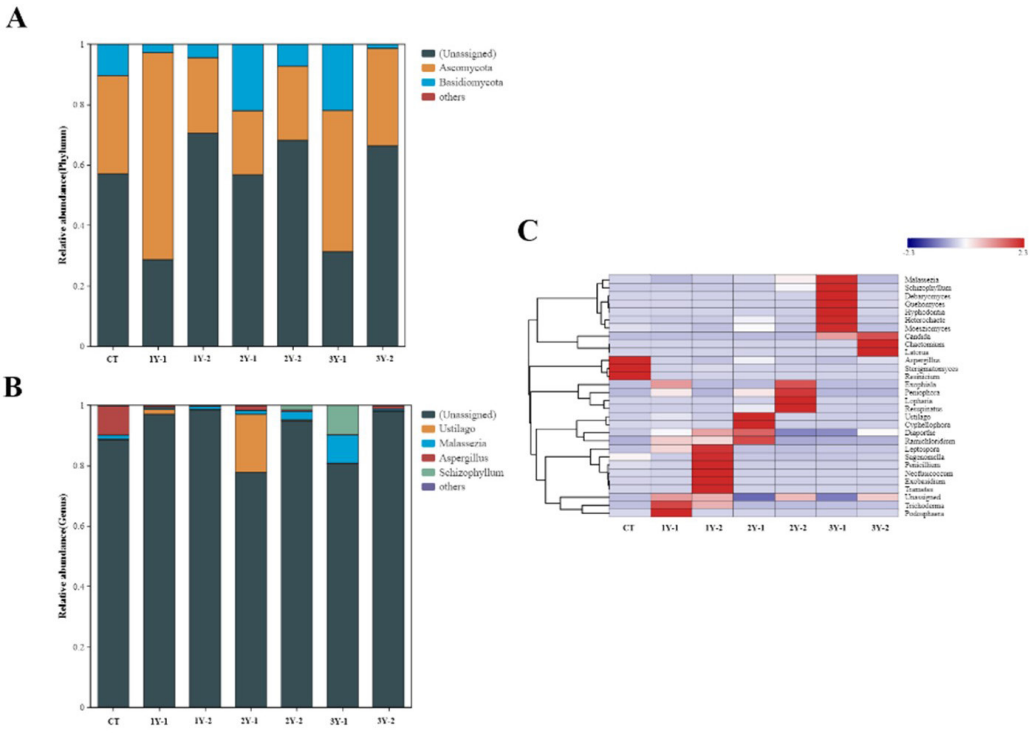

Figure 4. Fungal composition of LXH at phyla level (A) and genera level (B), and the cluster heatmap (C) during aging. The code refers to the collected sample time e.g., 1Y-1 refer to the sample collected in the early of first year and 1Y-2 refer to the sample collected in the later of first year. Other samples were named as well. The abscissa is the sample name and the ordinate is the relative abundance of difference fungi. Every column indicated a group different sample and every column contained different percent fungi with different color.

LXH during the aging time, which the contribution rate of the first principal component is 34.4%, the contribution rate of the second principal component is 15.1%, and the cumulative contribution rate is 49.5%. Similar to that of bacteria, the aging time also affects the bacterial colony of samples.

T test analysis of microorganism in LXH during aging time

The difference of microbial community between different samples of LXH was determined by Student's t-test analysis (Figures 6 and 7). A quantitative assessment of the evolution of the microbiome during aging time showed that the difference was generally consistent across the stages of aging time. A total of 20 different bacteria and 5 different fungi were found at different aging stages. The differences between bacteria are *Halomonas*, unassigned genera, *Nitriliruptor, Nesterenkonia, Weissella, Intestinibacter, Streptococcus, Stenotrophomonas, Pseudomonas, Nocardiopsis, Lactobacillus, Acinetobacter, Ralstonia, Gardnerella, Aquabacterium, Leuconostoc, Corynebacterium_1, Sphingomonas, Brevundimonas* and *Prevotella_7*. , especially in *Halomonas*, unassigned genera, *Nitriliruptor, Nesterenkonia, Weissella, Intestinibacter, Streptococcus* and *Nocardiopsis*, *Lactobacillus, Acinetobacter, Ralstonia*, while the change of *Nocardiopsis, Lactobacillus, Acinetobacter* and *Ralstonia* were decreased and then increased. The differences in fungi are mainly shown in unassigned genera, *Ustilago* spp. *Malassezia* spp. *Aspergillus* spp. and *Schizophyllum* spp., especially unassigned genera.

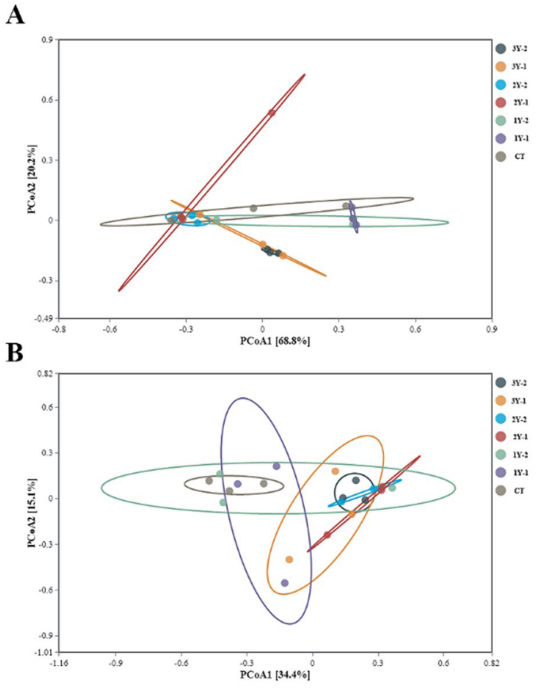

Figure 5. Principal co-ordinates analysis plots of LXH during aging based on bacterial OUT (A) and fungal OUT (B). The code refers to the collected sample time e.g., 1Y-1 refer to the sample collected in the early of first year and 1Y-2 refer to the sample collected in the later of first year. Other samples were named as well. Every circle indicated a group different sample with different color.

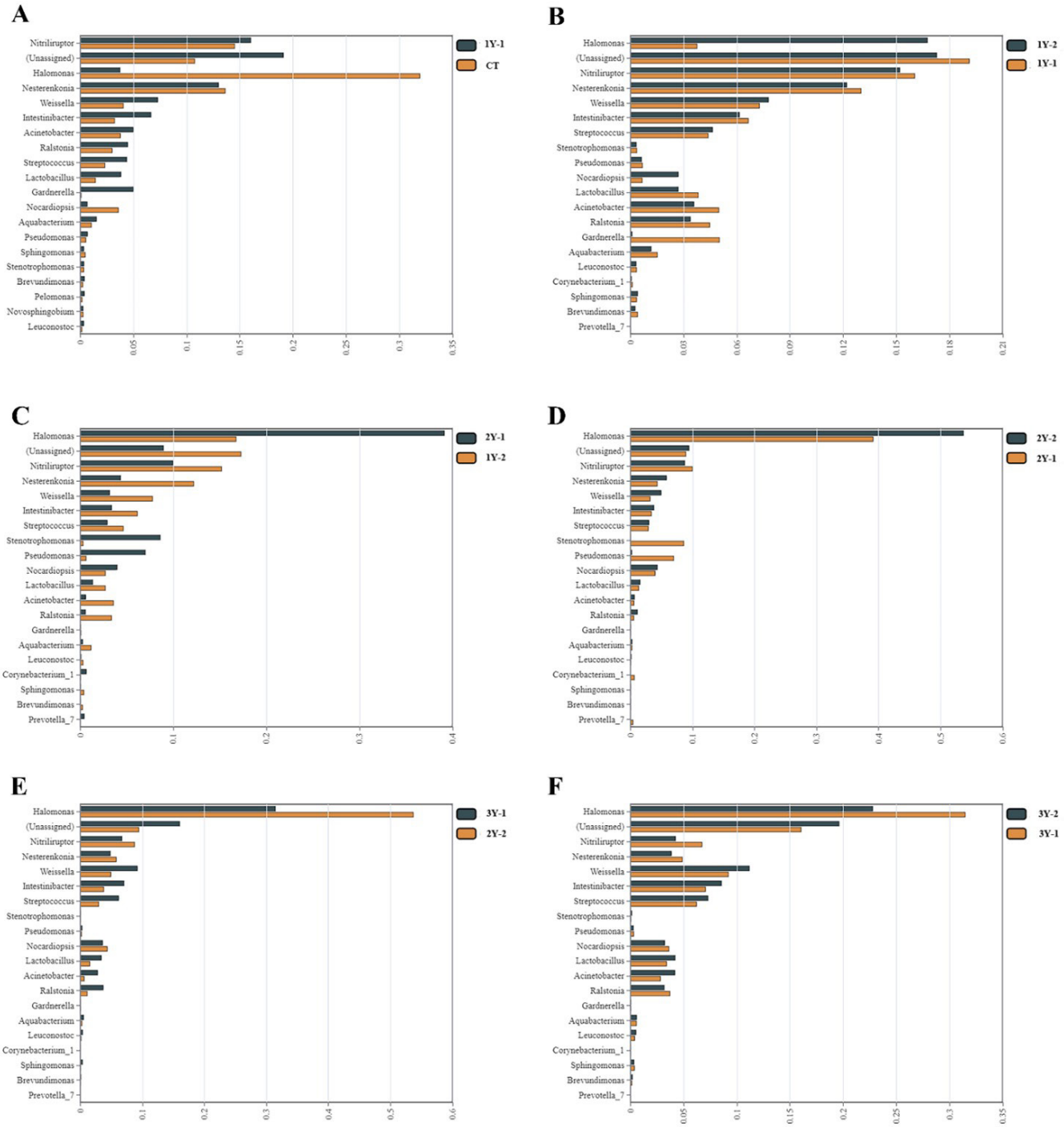

Figure 6. Bacterial student's t test of LXH between CT and 1Y-1 (A), 1Y-1and 1Y-2 (B), 1Y-2 and 2Y-1 (C), 2Y-1 and 2Y-2 (D), 2Y-2 and 3Y-1 (E), 3Y-1 and 3Y-2 (F) during aging, respectively. The code refers to the collected sample time e.g., 1Y-1 refer to the sample collected in the early of first year and 1Y-2 refer to the sample collected in the later of first year. Other samples were named as well. Every column indicated a group different microorganism and every column contained different percent with different color.

4 Discussion

LXH is a unique medicinal preserved fruit in Chao Shan area, China. This was the first study to investigate the changes in microorganisms that take place in aged LXH products The study provides a theoretical base for the swift development of LXH products.

High-throughput sequencing of bacterial and fungal amplicons showed an abundance and diversity of the microbiota

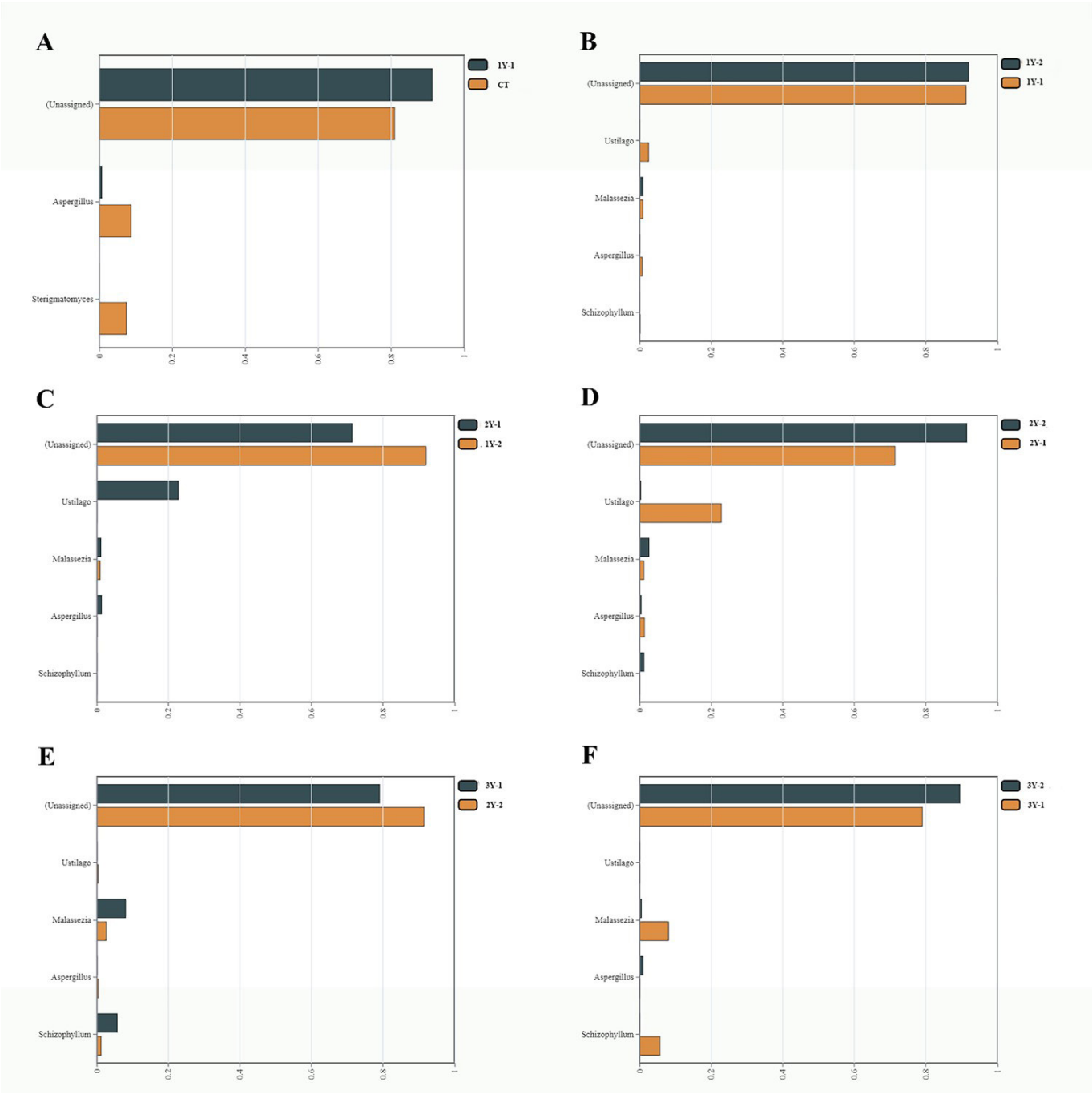

Figure 7. Fungal student's t test of LXH between CT and 1Y-1 (A), 1Y-1and 1Y-2 (B), 1Y-2 and 2Y-1 (C), 2Y-1 and 2Y-2 (D), 2Y-2 and 3Y-1 (E), 3Y-1 and 3Y-2 (F), during aging respectively. The code refers to the collected sample time, e.g., 1Y-1 refer to the sample collected in the early of first year and 1Y-2 refer to the sample collected in the later of first year. Other samples were named as well. Every column indicated a group different microorganism and every column contained different percent with different color.

in the LXH samples that were dramatically different among the samples during the different aging times. Alpha diversity has shown that the fungi decreased with aging time, while bacteria decreased first and then increased with the highest diversity appearing in the 1Y-1 and 3Y-2. According to the result of alpha diversity, PCOA analysis and T-test, the bacterial role is stronger than those of fungi. The evolution of bacteria was also significantly affected by the aging time more than that of fungi. Up to now, the fermented LXH is a spontaneous process without a starter. Thus, these complex bacterial communities may be derived from raw materials or processing environments. Nevertheless, LXH has lower microbial diversity than other fermented products, which may be due to the high sugar and salt content as well as the antibacterial properties of Citron (Xing et al. 2019). The environmental factors also resulted that the microbial composition of LXH mainly consists of

halophilic bacteria or halt-tolerant bacteria, such as (*Halomonas, Nitriliuptor, Stenotrophomonas, Pseudomonas, Debaryomyces, Sterigmatomyces,* etc. Some lactic acid bacteria (LAB) were also detected (*lactobacillus, streptococcus, weissella*, etc.). It is well known that LAB is not only considered as probiotics but also generates organic acids, ethanol and esters and other flavor substances through glycolysis, conducive to the fermentation of fruit and vegetable flavor (Pereira et al., 2018; Liu et al., 2019; He et al., 2021). Secondly, a certain LAB has also been confirmed to have biotransformation of active substances. The *Bacillus* sp. bio-converts the naringenin into eriodictyol via hydroxylation action (Chu et al., 2016). *Lactobacillus* and *bifidobacteria* with high levels of beta-glucosidase activity could hydrolyze tectoridin to more bioactive tectorigenin (Liu et al., 2012). *L. casei* decompose flavan-3-ols into m-coumaric acid, p-coumaric acid, 4-hydroxyphenylpropionic acid, and 3,4-hydroxyphenylacetic acid (Tabasco et al., 2011).

On the other hand, Halophiles commonly have a similar effect to LAB in fermented foods. *Halomonas* and *Pseudomonas* convert acetyl-CoA, acetyl benzoyl and carbamate to acetic acid by acetyl-CoA synthetase, and then use acetic acid to produce acetaldehyde and ethanol by aldehyde dehydrogenase (NAD +) (EC 1.2.1.3) and alcohol dehydrogenase (EC 1.1.1.1) (Liu et al., 2021). Furthermore, they also participate in the TCA cycle and the formation of lactic acid and acetic acid, which helps its unique acidity, taste and flavor of fermentation (Xiao et al., 2018). In addition, *Halomonas* also possesses 4-aminobutyric aminotransferase (EC 2.6.1.19), which is associated with the production of gamma-aminobutyric acid (GABA) (Liu et al., 2021). During the aging time of Chen Pi, *Pseudomonas, Bacillales* and *Enterococcus* were found to significantly connect with the change of Nobiletin (He et al., 2019).

Although the abundance of fungi is low, they are an important part of the aging process. During the aging process, *Aspergillus* niger and *Schizophyllum* spp. have been proven to transform flavonoids to increase their content of hesperidin, nobiletin, and total flavonoids or produce new chemical compounds with antioxidant and expectorant properties (Wu et al., 2012; Wang et al., 2015, 2018; Liu et al., 2017). *Aspergillus niger* has succeeded in shortening the aging cycle of Chen Pi (Wang et al. 2020).

In addition to the biotransformation of microorganisms, the products secreted by some microorganisms themselves are also of interest. *Halomonas*, *Lactobacillus*, *Bacillus* and *Ralstonia* have been reported that they can generate polyhydroxybutyrate (PHB), a 100% biodegradable thermoplastic discovered by Lemoigne in bacteria in the early 1920s, which is similar to polypropylene (PP) (Tohyama et al., 2000; Sangkharak & Prasertsan, 2007; López et al., 2012; Guo et al., 2020). However, it has been reported that the intestinal flora of animals can decompose the PHB into short-chain fatty acids monomers (Sui et al., 2012). The monomers not only provide energy for the growth of intestinal flora, but also create an acidic environment that inhibits the growth of harmful bacteria (Baruah et al., 2008). PHB can also alter the intestinal flora structure and promote the proliferation of beneficial bacteria (Baruah et al., 2008). Currently, PHB is mostly used in aquaculture, but not in other fields (Gao et al., 2019a). As well, *Halomonas* spp. and *Schizophyllum* spp. can produce polysaccharides and fructans (Gao et al., 2019b). Exopolysaccharide has anticancer and antioxidative properties, as well as being prebiotic that can support the growth of beneficial microbes and inhibit the growth of pathogens in food (Okamura-Matsui et al., 2001). *Nitriliuptor* spp. is a member of the actinomycetes, which are capable of synthesizing carotene (Leplat et al., 2019). This might explain the color change of LXH with age. *Nesterenkonia* spp. has the potential to produce oligosaccharides and xylooligosaccharides due to its alpha-amylase and xylanase abilities (Ojha et al., 2015; Kui et al., 2010). *Stenotrophomonas* spp., *pseudomonas* spp., and *streptococcus* spp. have been shown to produce oligosaccharides with prebiotic effects and degrade aflatoxin (Yamashita et al., 1988; Abou-Taleb & Galal, 2018; Cai et al., 2020). *Nocardiopsis* spp. secretes alpha amylase. It has the potential to produce oligosaccharides (Chakraborty et al., 2014), and can secrete antimicrobial peptides (Joseph et al., 2021).

5 Conclusion

A total of 13 bacterial genera and 4 fungal genera were identified in LXH. Each genus has its own unique function in aging process. Among them, bacteria, specially *halomonas* spp. *Nitriliuptor* spp., *Stenotrophomonas* spp. plays a major role in the aging process, which has the potential to promote the production of functional components of LXH, including providing beneficial exopolysaccharides and prebiotics. Whereas microorganisms found in LXH have several benefits, there are also significant risks related to microbial contamination. Among other things, the fungus *Aspergillus niger* causes foodborne toxin and *Schizophyllum* spp. causes harmful infections, while *Malassezia* spp. can lead to pityriasis versicolor, Malassezia folliculitis and seborrheic dermatitis. Therefore, it is impossible to ignore the potential harm caused by these microorganisms.

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Supplementary Material

Supplementary material accompanies this paper.

Table S1. The alpha diversity of microorganism in LXH during aging time

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