Food Research International 156 (2022) 111126

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Spatiotemporal distribution of environmental microbiota in spontaneous fermentation workshop: The case of Chinese Baijiu

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ARTICLE INFO

Keywords:
Environmental microbiome
High-throughput sequencing
Microbial source tracking
Chinese Baijiu
Spontaneous fermentation

ABSTRACT

The strong-flavor Baijiu (SFB) brewing workshop is a complex ecosystem with diverse microbiomes. As a potential source of microbiomes in fermentation, microbiota in the environmental microecology may affect the quality and flavor of SFB. Here, we report the collection of environmental microecological samples from three SFB workshops with different usage times (named 70a, 30a, and new, respectively). We used 16S rRNA and internal transcribed spacer (ITS) gene amplicon full-length sequencing to explore the microbial community structure in SFB. The SourceTracker tool was used to investigate links among fermentation samples, raw materials, and the environment and decipher the construction process in the workshop indoor environment. *Lactobacillus acetotolerans* was the most important bacterial genus in Zaopei after fermentation, whereas other types of samples exhibited different prokaryotic community structures. The composition of the fungal community was similar, with *Saccharomycopsis fibuligera*, *Debaryomyces hansenii*, *Lichtheimia ramosa*, *Lichtheimia corymbifera*, and *Pichia kudriavzevii* being the most abundant, and were detected in most samples. Further comparison of the microbiota in the workshop environment showed that the diversity of the microbiota in the indoor environment decreased, showing different clustering patterns under the influence of location. With increasing usage time, the contribution of deterministic processes to the assembly of the prokaryotic community increases, and the community structure tends to stabilize, exhibiting its own characteristics. SFB-fermenting resident functional fungi were the major components of the fungal community, and SourceTracker analysis also highlighted the contributions of Zaopei, Daqu, and tool surfaces as fungal sources. This study is the first to comprehensively monitor the microbial profile of the SFB production environment. This research can be extended to involve more complex spontaneous fermentation environment microbiota and has important implications for the control of spontaneous fermentation.

1. Introduction

Strong-flavor Baijiu (SFB), also called "Luzhou-flavor Baijiu," is a traditional spontaneously fermented food with an ancient history, produced by distilling mixed fermented grains (Zaopei). SFB is popular among consumers because of its unique flavor sensory properties, thus dominating the food market (Jin, Zhu, & Xu, 2017). SFB production involves fermentation with microbiota from raw materials and complex environments, which have a direct impact on the quality and flavor of fermented foods (Coller et al., 2019). Unlike modern food factories, strict cleaning and disinfection are required to maintain a sanitary indoor environment (Zwirzitz et al., 2020). There is less manual

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https://doi.org/10.1016/j.foodres.2022.111126
Received 18 November 2021; Received in revised form 8 March 2022; Accepted 9 March 2022
Available online 12 March 2022

intervention in the spontaneous fermentation workshop, and the open-operation process provides conditions for the enrichment of microbiomes in the environment, while providing the potential for microbial exchange between the environment and fermented foods (De Roos, Van der Veken, & De Vuyst, 2019; Johnson, Curtin, & Waite-Cusic, 2021). According to previous studies, microbiota from the environment is involved in the fermentation process of Baijiu and could drive both microbial succession and metabolic profiles (Pang et al., 2018; Wang, Du, Zhang, Xu, & Björkroth, 2018). However, to the best of our knowledge, no study has comprehensively investigated microbial populations in SFB processing environments.

The microbiomes of Zaopei, Daqu (DQ, the starter for fermentation), and pit mud (PM), which are important for SFB fermentation, have been deeply analyzed using high-throughput sequencing methods, showing that a variety of microorganisms can work together to participate in Baijiu fermentation and flavor-compound production (Fu et al., 2021; Hu et al., 2021; Mao et al., 2022; Wang, Du, & Xu, 2017). Meanwhile, the results also showed that the composition of PM in the microbial community was complex (Liu et al., 2017, 2020; Tao et al., 2014). With increase in usage time, the microbial diversity of PM increased, and the community structure was transformed into more abundant functional eubacteria and archaea, dominant in mature PM (Zhang et al., 2020, 2015). The positive succession of PM microbiota in the time series gives us reason to believe that the construction process of the environmental microbiota in the workshop is regular. However, current research has pointed out that long-term continuous production has a limited impact on the changes in environmental microbial communities. Nevertheless, there has been a lack of exploration of the temporal and spatial succession of environmental microbial communities. To explore the temporal and spatial succession of the SFB microbial community, researches mostly focus on short-term stages, such as Zaopei fermentation and DQ maturation (Du, Wang, Zhang, & Xu, 2019; Liu et al., 2021; Zhang et al., 2021); meanwhile, a thorough understanding of the succession of the environmental microbial community in the brewing workshop remains lacking.

To analyze the microbial population structure of the SFB brewing environment and explore succession in the SFB brewing environment in terms of time and space, three SFB workshops with different usage times (70a, 30a, and new workshop) were sampled and analyzed using single-molecule real-time (SMRT) sequencing. The microbiota of the brewing environment microecology in the SFB workshop was systematically characterized, and the contribution of the brewing process environment to SFB fermentation was analyzed. The diversity of the internal environmental microbiota community in the SFB workshops with different usage times was compared, and the law of succession of the microbial community structure in the brewing environment was explored with regard to time and space. Finally, the construction process of the indoor environment microbial community was investigated using the SourceTracker tool. This work provides an in-depth understanding of the environmental microbial composition in SFB production workshops and new insights into the environmental microbial community of traditional fermentation production workshops.

2. Materials and methods

2.1. Sampling site

The experimental site was located at the famous SFB enterprise in Luzhou, Sichuan Province, China (28° 91′ N; 105°39′ E). In October 2020, we collected environmental samples in from three SFB fermentation workshops (70a, 30a, and new). The 70a workshop was built in 1573 and has been renovated many times; the last renovation was carried out in the 1950s. The workshop uses traditional mud pit pits for fermentation, all of which have been used for more than 100 years, including one which has been continuously brewed since 1573 CE (Zhao, Zhang, & Zhou, 2009). The 30a workshop was built in 1986 and has been in use for more than 30 years. The new workshop has been used for Baijiu fermentation since the completion of its construction in 2019. Both the 30a and the new workshops used stainless-steel wine cellars for fermentation.

2.2. Sample collection

All samples were obtained from the 70a, 30a, and new workshops for the SFB product. The samples consisted of fermented grains (Zaopei), raw materials, and processed environmental specimens. Details regarding these samples are provided below and are summarized in Table 1.

The production of SFB mainly involves steaming and cooling the grains (sorghum and rice husk), mixing them with DQ powder, and then placing the mixed grains (Zaopei) into a fermentation cellar (traditional underground mud or stainless-steel cellars). After fermentation, the Zaopei was added to distilled water to collect liquor. In the different workshops with different usage times, pits were randomly selected to collect the pre-fermentation Zaopei (BZ), fermented Zaopei (EZ), and PM samples. For the EZ and PM samples, the location of the sampling points in the pit should be considered; the specific sampling point positions are represented in Table S1.

The tool surfaces (TS), hand surfaces of workers (HS, traditional 70a workshops), and equipment surfaces (ES, 30a, and new workshops) were wetted and sampled, using sterile absorbent cotton pre-wetted with sterile phosphate-buffered saline (0.1 M). Multiple randomly sampled bags for the raw materials of rice husk, sorghum, and DQ, obtained from each workshop, were mixed at multiple points as a sample.

Indoor environmental samples included doorway ground (DG), indoor ground (IG), indoor walls (IW), and indoor air (IA). DG, IG, and IW samples were collected from ground and wall sediments, sampled at multiple locations in the workshops, and mixed into one sample. A Total Suspended Particle High-Volume Sampler was used to collect IA samples. The sampling membrane was made of glass fiber (25 mm, Pall Life Sciences, USA), and the sampling flow rate was set to 1.05 m^3/min. The sampling time for each sample was 6 h, and three parallel samples were collected from each workshop. Outdoor environmental samples were taken from soil far away from the workshop (OG) and from the non-flowing air (OA) around the workshop (surfaces of plants around the workshops). OA samples were also obtained by wiping leaf surfaces with moistened sterile absorbent cotton.

With the exception of IA, at least four parallel samples were collected for the sample types, along with the sample codes and quantities (Table S1). After sampling, all samples were placed in a −80 °C freezer and transported back to the laboratory by cold-chain transportation.

Table 1
Description of the samples used in this study.

	Sample code	Sample type
Zaopei (grains mixed with Daqu powder)	BZ	Unfermented Zaopei
	EZ	Fermented Zaopei
Contact surface	PM	Pit mud
	TS	Tool surface
	HS	Hand surfaces of workers in 70a workshop
	ES	Equipment surface in 30a and new workshops
Raw material	DQ	Daqu (Fermentation Starter)
	R	Rice husk
	S	Sorghum
Indoor enviroment	IG	Indoor ground
	IA	Indoor air
	IW	Indoor wall
	DG	Doorway ground
Outdoor enviroment	OG	Soil far away from the workshop
	OA	Non-flowing air around the workshop (surface of plants around workshops)

2.3. DNA extraction, amplification, and sequencing

Total DNA was isolated using the Fast DNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA), according to the manufacturer's protocol and stored at −80 °C. For bacteria, the 16S rRNA gene was amplified using the universal primers 27F_ 1492R (5′-AGRGTTTGATYNTGGCTCAG-3′/5′-TASGGHTACCTTGTTASGACTT-3′) with the barcode. For the fungi, the internal transcribed spacer (ITS) region was amplified using barcoded universal primer ITS1_ITS4 (5′-CTTGGTCATTTAGAGGAAGTAA-3′/5′-TCCTCCGCTTATTGATATGC-3′). Polymerase chain reaction (PCR) products were purified using a PCR purification kit, and the concentrations were carefully assessed by the Thermo Scientific NanoDrop 8000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The SMRT Bell library was built as required and then purified by AM Pure PB beads (Pacific Biosciences, Menlo Park, CA, USA). Furthermore, an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) was used to detect the size of the library fragments. Sequencing was performed using the PacBio Sequel II instrument.

2.4. Bioinformatics

The original image data obtained by sequencing were converted into sequence data by base calling and stored in the BAM (Binary Alignment/Map format) file. For quality control of the sequencing data, the Arrow algorithm was used to obtain high-accuracy raw CCS sequences. The raw CCS sequence of each sample was obtained using Lima software to identify different Barcode sequences. Sequences with an accuracy of more than 99% were selected and BLAST software was used to match and intercept the target product sequence (preserve the primer region by default) and correct the sequence direction. For 16S rRNA CCS reads, only sequences greater than 1500 bp in length were chosen for further analysis. ITS CCS reads smaller than 600 bp were removed prior to downstream analyses. Chimeric sequences were removed using the UCHIME algorithm (Edgar, Haas, Clemente, Quince, & Knight, 2011). From this technique, clean CCS data for each sample were obtained.

2.5. Data analyses and statistics

All clean CCS sequences were further processed using QIIME (v1.9.1) (Caporaso et al., 2010). The unique sequence set was classified into operational taxonomic units (OTUs) based on a 97% threshold identity using UCLUST (Edgar, 2010). A single representative sequence from each clustered OTU was aligned to the Silva database (v13.2) (DeSantis et al., 2006) and UNITE fungal ITS database (v12.11) (Abarenkov et al., 2010) to obtain classified information. Before further analysis, singleton OTUs were removed. The Chao1 richness index and Shannon diversity index were calculated using QIIME (Caporaso et al., 2010). Nonmetric multidimensional scaling (NMDS) provides visualization of the microbial community composition. Similarity analysis (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2010) were used to determine the differences in microbial communities. To identify the microbial biomarkers in different workshop environments, we used the Galaxy Web application (https://huttenhower.sph.harvard.edu/galaxy/) to calculate the linear discriminant analysis (LDA) effect size (Segata et al., 2011). The species-level clustering results for each sample were used as inputs for the LEfSe analysis. The Kruskal–Wallis test was used to determine the difference between the categories, a significance level of $p < 0.05$, and a threshold LDA score of 4.0.

Microbial source tracking was achieved using SourceTracker software (version 1.0.0) and default parameters (Knights et al., 2011). OTU tables were used as data input for modeling using the "SourceTracker" R package (https://github.com/danknights/sourcetracker). The outdoor environment, raw materials, Zaopei, and contact surface samples of each workshop were set as "sources"; and the indoor environmental samples of each workshop were set as "sinks".

We used the microbial community construction parameter, beta nearest taxon index (βNTI) based on null-model theory (Dini-Andreote, Stegen, van Elsas, & Salles, 2015) to characterize and evaluate the assembly process of the microbial community in the indoor environment of the three workshops. The picante package (Kembel et al., 2010) in R was used for calculations (Edwards, Santos-Medellín, & Sundaresan, 2018), in which βNTI value between −2 and + 2 indicates the dominance of stochastic processes and βNTI value greater than + 2 or lower than −2 indicates the dominance of deterministic processes.

3. Results

3.1. Microbial landscape of the SFB workshops

To explore the microbial composition and distribution in the microecology of SFB workshops, we obtained samples from three workshops with different usage times, a total of 241 samples were collected for PacBio full-length high-throughput sequencing. The rarefaction curves of both prokaryotic and fungal communities approached the saturation plateau, which indicated that the microbial communities were well represented at the sequencing depth (Fig. S1).

Regarding 16S rRNA gene sequencing data, a total of 4,493,556 high-quality reads (average length of 1,528 bp) were obtained. After removing chimeras and singletons, 2,127,860 reads were clustered into 43,634 OTUs based on 97% similarity. According to the database alignment results, 82.96% of the 16S rRNA gene reads from the three SFB workshops belonged to 33 bacterial phyla and 2 archaeal phyla, and the remaining sequences were identified as unassigned reads because they could not be found in the database. As shown in Fig. S3, Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria were the main bacterial populations detected in all the samples. Regarding Archaea, Euryarchaeota was the most important phylum, mainly detected in the PM samples from the 70a and 30a workshops, and was also found in the Zaopei, TS, and ES samples, which were in direct contact with PM. The relative abundances of the phyla showed different patterns according to different sample types: Firmicutes became the dominant phylum after Zaopei fermentation, and was also detected in TS, HS, and ES in large quantities. In the workshop environmental samples, unassigned bacteria accounted for a large proportion, whereas Proteobacteria was the dominant phylum.

At the genus level, 874 prokaryotic genera were detected, of which six genera were from Archaea. Based on the relative abundances of the genera observed among the samples (Fig. 1), *Methanobrevibacter* was the most abundant archaeal genus, which was mainly detected in the PM samples (24.36%) of the 70a workshop. It was also detected in both contact surface samples from the 70a and 30a workshops. After fermentation, the main bacterial genera in EZ were *Lactobacillus* (70.62%–79.40%) and *Acetilactobacillus* (2.53%–7.56%). *Prevotella 7* (9.33%) was also detected in the 70a EZ samples, whereas *Prevotella 7* appeared in the 70a workshop BZ (9.33%) and PM (6.21%) samples and in the 30a workshop PM (11.03%) and IG (17.51%) samples. Only a small amount of *Prevotella 7* was detected in the new workshop. BZ samples from different workshops also showed different compositional patterns; the main bacterial genera included *Acetobacter*, *Lactobacillus*, and *Bacillus*. In addition, *Lysinibacillus* (25.52%) from 70a workshop, *Thermoactinomyces* (41.24%), and *Pseudomonas* (16.74%) from 30a workshop were the dominant genera of BZ samples, respectively. For the contact surface samples (HS, TS, and ES), *Staphylococcus* (6.51%–65.79%) and *Lactobacillus* (5.26%–65.22%) were the dominant genera. The starter sample DQ used in the different SFB workshops also showed different bacterial compositions. *Bacillus* and *Saccharopolyspora* showed higher relative abundances in the DQ samples from 70a and 30a workshops, whereas the main genera of DQ in the new workshop were *Pseudomonas*, *Staphylococcus*, *Weissella*, and *Thermoactinomyces*. Indoor environmental samples contained a rich variety of prokaryotic

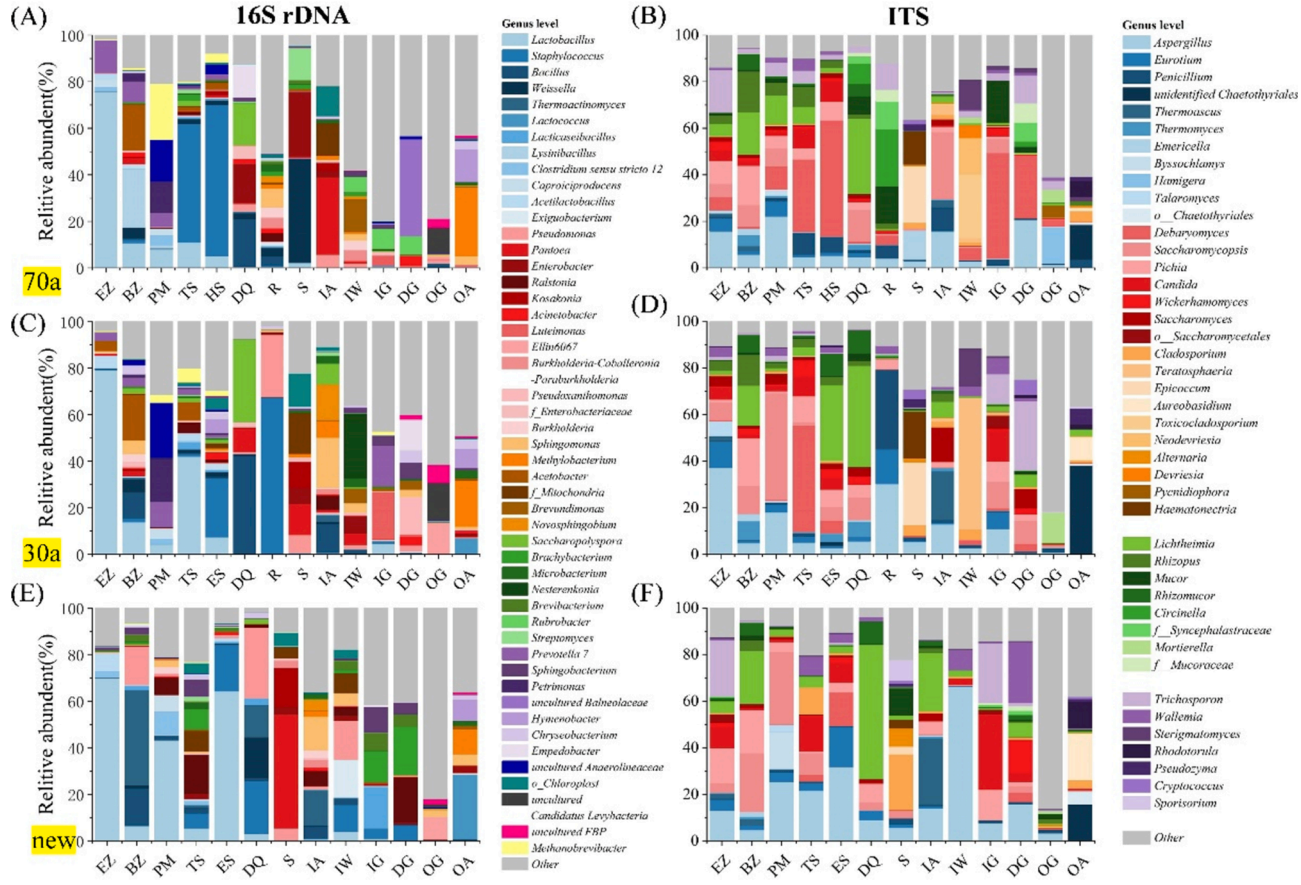

Fig. 1. Compositions of the microbial communities of the SFB workshops at the genus level. (A), (C), (E) Prokaryotic community composition of the 70a, 30a, and new workshops. (B), (D), (F) Fungal community composition of the 70a, 30a, and new workshops. Taxa compromising < 0.5% of the total relative abundance across all samples are grouped as others.

microorganisms. It is worth noting that the SFB workshops showed unique microbial characteristics. *Rubrobacter* was the main genus in the ground and wall samples of the 70a workshop. Meanwhile, in the new workshop, *Ralstonia* had higher relative contents in the PM (7.71%), TS (17.10%), IA (6.45%), and DG (20.26%) samples.

Lactobacillus acetotolerans was the most important *Lactobacillus*, and the remainder was mostly uncultured *Lactobacillus sp*. *Lactobacillus acetotolerans* mostly appeared in the Zaopei and contact surface samples and was only slightly detected in the workshop environments, except in the new workshop IW samples. In the new workshop, *Thermoactinomyces vulgaris* (16.20%), *Thermoactinomyces intermedius* (14.34%), and *Bacillus licheniformis* (14.53%) dominated the BZ samples. In addition, *T. vulgaris* and *T. intermedius* were also detected in the DQ and IA samples (Fig. 2).

ITS gene sequencing yielded 3,072,015 high-quality reads (average length of 734 bp). After passing quality filtering, the reads were clustered into 69,891 OTUs, of which 71.21% of the reads belonged to Ascomycota, Zygomycota, Basidiomycota, Blastocladiomycota, Chytridiomycota, and Glomeromycota; the remaining sequences were classified as "unassigned" (Fig. S4). Among the assigned divisions, Ascomycota, Zygomycota, and Basidiomycota were detected in all samples, whereas Blastocladiomycota, Chytridiomycota, and Glomeromycota were found only in the environmental samples, that too in less abundance.

In total, 423 fungal genera were detected at the genus level. *Aspergillus*, *Lichtheimia*, *Debaryomyces*, *Saccharomycopsis*, *Pichia*, *Trichosporon*, *Candida*, and *Eurotium* were widely detected in Zaopei, contact surfaces, raw materials, and the indoor environments of the workshops (Fig. 1). *Aspergillus* abundance increased during the fermentation process of Zaopei and became the main fungus in the contact surface and ground samples, especially on the contact surface (PM, 25.39%; TS, 21.55%; ES, 31.83%) and walls (IW, 66.17%) in the new workshop. *Lichtheimia* was the predominant fungus in DQ (32.33%–57.49%) and was also present in the BZ samples (17.12%–32.58%), with the content of *Lichtheimia* decreasing as fermentation progressed. In the 70a workshop, *Debaryomyces* was detected in large quantities on the ground (IG, 45.42%; DG, 26.53%) and contact surfaces (PM, 10.07%; TS, 31.09%; HS, 49.81%).

Focusing on the distribution of fungal species, as shown in Fig. 3, some of the most dominant fermentation fungi were *Saccharomycopsis fibuligera*, *Debaryomyces hansenii*, *Lichtheimia ramosa*, *Lichtheimia corymbifera*, *Pichia kudriavzevii*, *Aspergillus penicillioides*, and *Aspergillus chevalieri*. They were widespread among the indoor environmental (especially PM, TS, HS, and ES) and raw material samples.

3.2. Microbial diversity of the SFB workshops

Alpha-diversity indices (Chao1 for richness, Shannon for evenness) (Fig. S2) showed significant differences between the outdoor environment samples (OD and OA) and the microecological microbial communities inside the workshops. The alpha-diversity indices of OD and OA samples were higher than those of the other sample types. For the indoor microecological samples, only the prokaryotic community of the indoor environment (including IA, IW, IG, and DG) showed a significantly higher Shannon index compared to the Zaopei, raw material, and contact surface samples. The results of the rest of the alpha-diversity analyses did not reveal any significant differences among the different samples.

To assess the microbial beta diversity in the microecological environment of the SFB workshop, NMDS analysis was used to visualize the

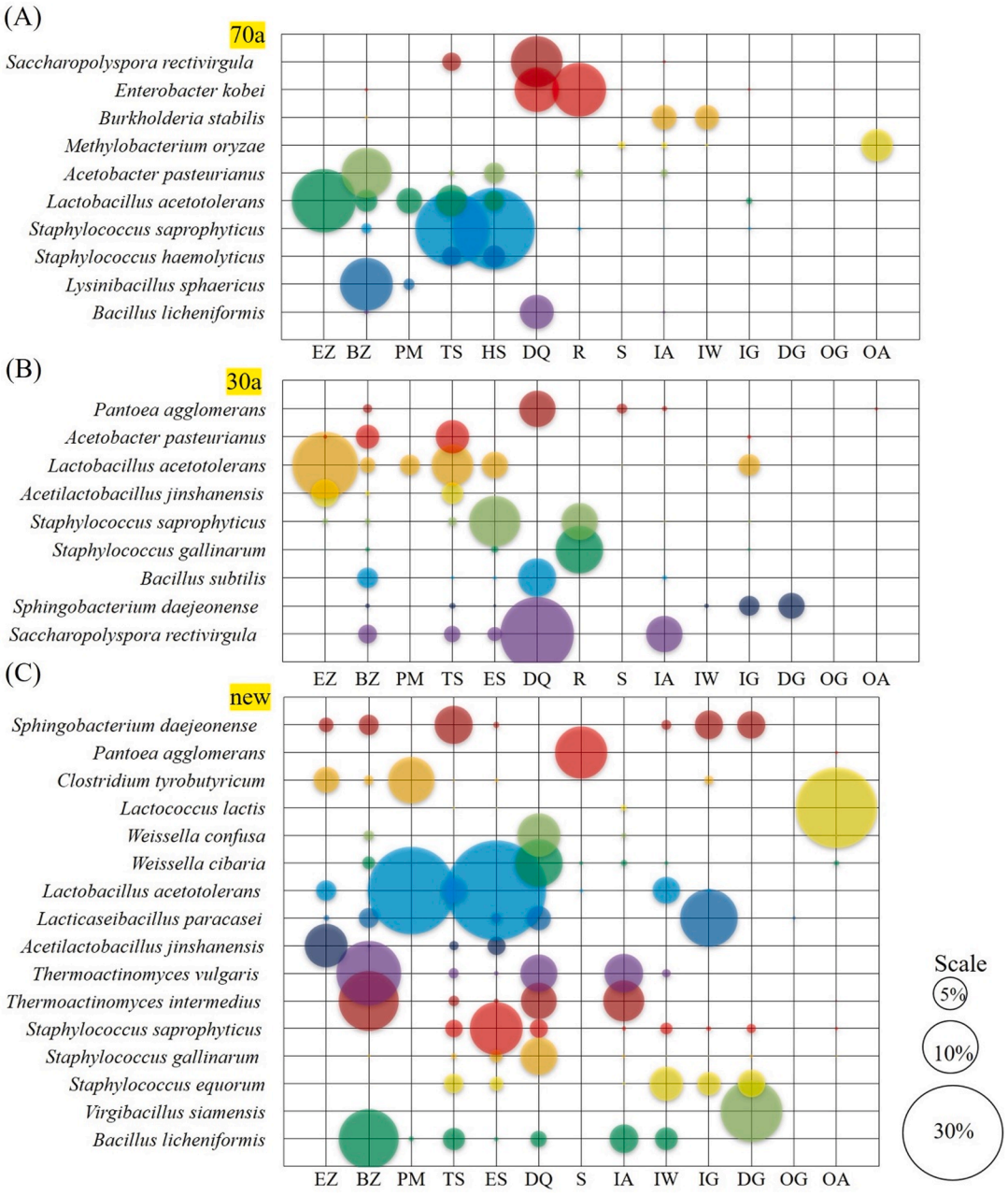

Fig. 2. Bacterial species in the (A) 70a, (B) 30a, and (C) new workshops. Only showing genera with relative content ≥ 1% in at least one type of sample.

differences in community structure (Fig. 4). Based on 97% similarity at the OTU level, the diversity of the microecological environment samples of the 70a, 30a, and new workshops was analyzed. The microbial populations of the different types of samples showed significant differences ($p = 0.01$), with the microbial community structure of the outdoor environment showing a clear separation from that of the interior of the workshop, especially in the new workshop. The microbial classification patterns of the workshops with different usage times were slightly different, whereas the prokaryotic community exhibited greater location differentiation. With increase in usage time, the sample points in the 70a workshop were clearly separated based on the sample types. The sample points of the fungal community in the workshop showed a partial overlap, indicating that the fungal composition of the internal environment of the workshop was similar to that of the raw material and Zaopei, with a relatively close distance.

3.3. Microbial community differences in the indoor environment

Further analysis of the microbial community in the indoor environment (including DG, IG, IW, and IA) was carried out to explore the succession of microorganisms in the SFB brewing environment during the time series. Alpha-diversity analysis (Table. S2) showed that the alpha diversity of the bacteria communities in the IA and IG samples of the 70a workshop was significantly higher than that in the other two workshop samples ($p < 0.05$), whereas the diversity and richness of the fungal communities were not significantly different.

PERMANOVA indicated that the main part of the variance of the microbial community in the indoor environment was explained by the different location types ($R^2_{Bacteria} = 0.2043$; $R^2_{Fungi} = 0.1980$, $p = 0.001$), whereas the different usage times of the workshop helped explain the smaller part of the overall variance ($R^2_{Bacteria} = 0.1309$; $R^2_{Fungi} = 0.1344$, $p = 0.001$) (Table.2). The differences in microbial communities were affected by the type of location. According to the

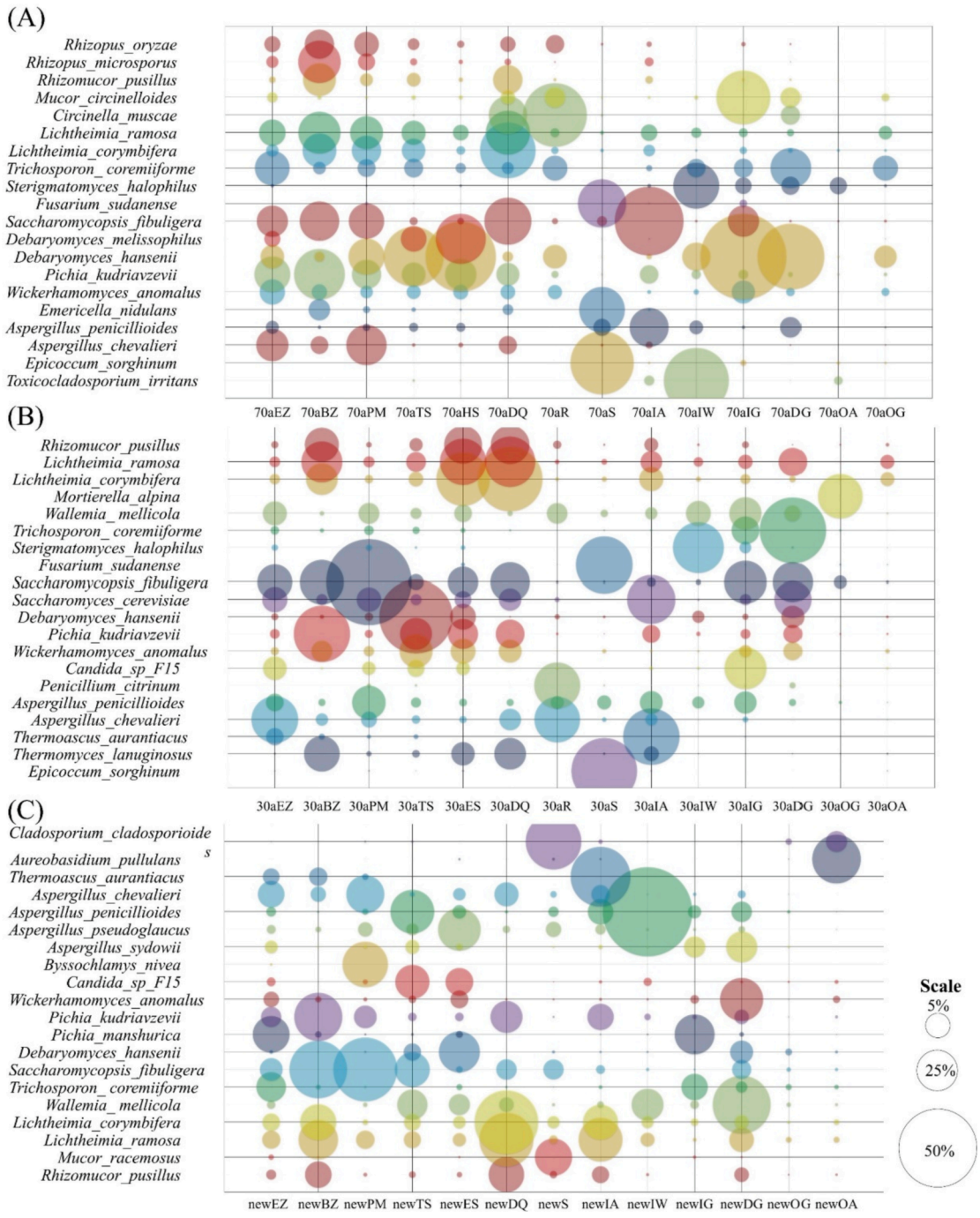

Fig. 3. Fungal species in the (A) 70a, (B) 30a, and (C) new workshops. Only showing genera with relative content ≥ 1% in at least one type of sample.

relative abundances of OUTs, the NMDS analysis results (Fig. 5) also showed that there were significant differences in the microbial community structure at different sampling locations in the three workshops (ANOSIM; Bacteria, $R = 0.7217$, $p < 0.001$; Fungi, $R = 0.7315$, $p < 0.001$). For bacterial communities, a more obvious classification pattern was observed; ground samples (DG and IG) from the same workshop demonstrated good clustering properties. The 70a DG and IG samples were significantly different from the other samples, indicating that the structure of the planting microbial community on the ground changed steadily as the usage time increased. For fungi, IA samples from the three workshops showed obvious clustering results, which were similar to the clustering results of the IW samples of the respective workshops.

LEfSe was used to detect microbial biomarkers in the indoor environments of the SFB workshops; the results of the differing species with LDA greater than 4 are shown in Fig. 6. For ground samples, the 70a and new workshops showed significant differences, with the biomarkers as follows: 70a = *Rubrobacter*, *Erythrobacter*, *Debaryomyces hansenii*, and *Aspergillus sclerotiorum*; new = *Brevibacterium*, *Brachybacterium*, *Staphylococcus equorum*, and *Candida*. The IW samples of the three workshops exhibited characteristics different from other samples; *Bacillus licheniformis* and *Aspergillus penicillioides* were the dominant species in the new workshop. For IA samples the content of *Aspergillus chevalieri* and *Bacillus licheniformis* in the new workshop was significantly higher than that in the other workshops.

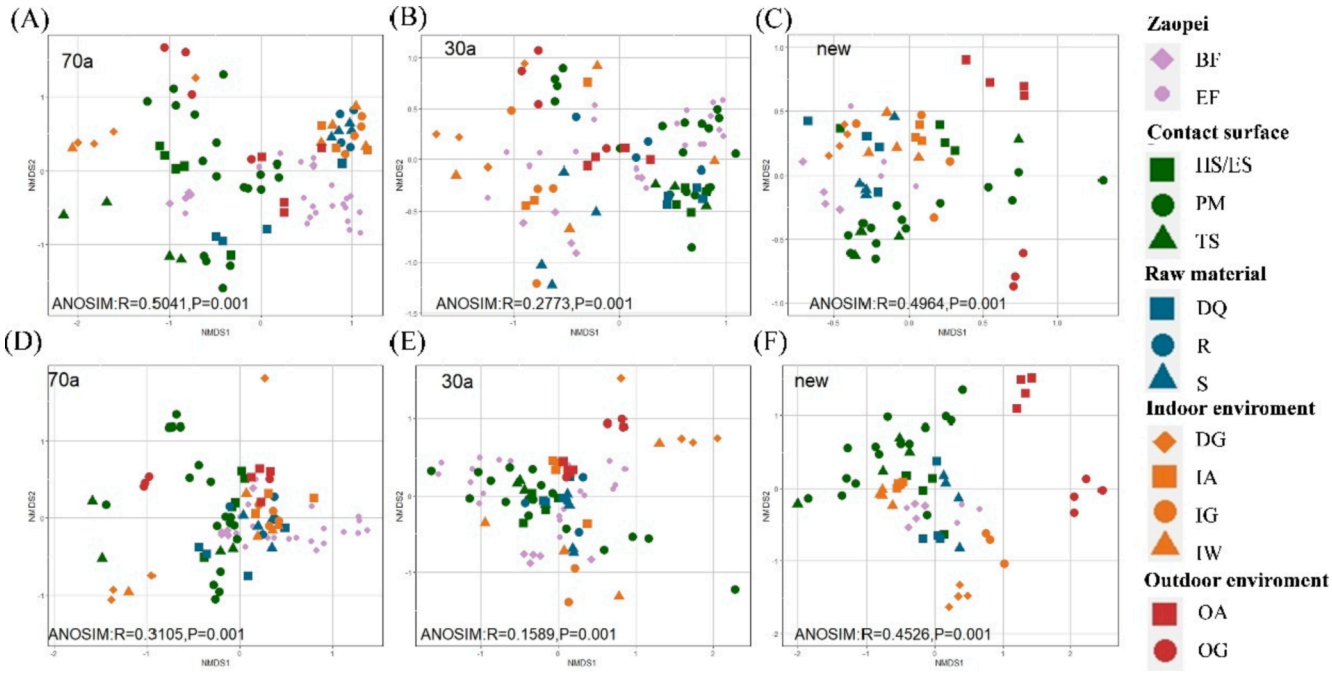

Fig. 4. Non-Metric Multidimensional Scale (NMDS) analysis of prokaryotic and fungi in the microecology of (A, D) 70a, (B, E) 30a, and (C, F) new workshops. ANOSIM is used to analyze the effects of different samples on bacteria and fungi. All analyses are based on the relative abundance of OTU levels.

Table 2
Permutational MANOVA Comparisons of Bray-Curtis Dissimilarity (BC) Microbial Diversity in different workshops insides environments.

Explanatory variable	Bacteria R^2	*P*	Fungi R^2	*P*
Usage time (70a, 30a, new)	0.1309	0.002	0.1344	0.001
Site (DG, IG, IW, IA)	0.2043	0.001	0.1980	0.001

R^2 is the variance contribution, indicating the degree of explanation of the difference between different groups, that is, the ratio of the grouping variance to the total variance. The larger R^2, the greater the grouping explanation of the difference; P, the significance p value, the default $p < 0.05$ means existence significant differences.

3.4. Source and construction of the microbial community in SFB workshops

To further insight the time series succession of the environmental microbiome (including DG, IG, IW, and IA) in the SFB workshop, we explored the source and assembly process of environmental microbiome. 10 potential sources (including Zaopei, contact surface, raw material, and outdoor environment samples) were quantitatively analyzed using SourceTracker. The results are shown in Fig. 7, illustrating that there were differences in the main sources of the environmental microbiomes among the different workshops. As opposed to the bacteria, the fungal communities in the environments were associated with more definitive sources. In the 70a and 30a workshops, the IG, DG, and IW sources of most of the bacteria were classified as "unknown." The main sources of fungi on the ground in the 70a workshop were rice husk (15.75%–20.28%) and HS (14.78%–32.18%). For the 30a workshop, PM

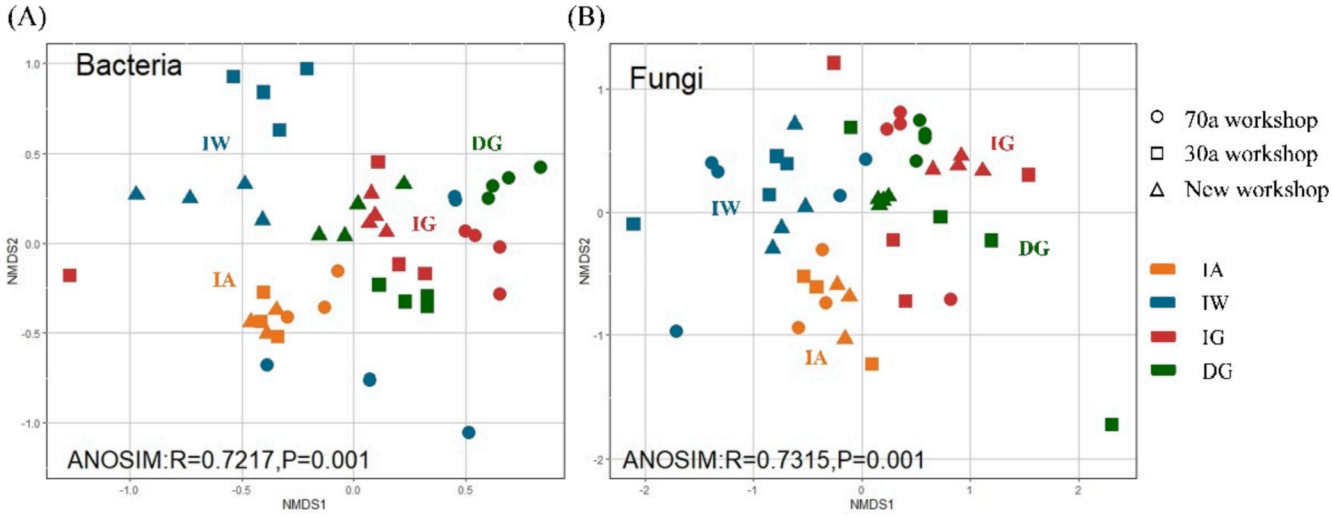

Fig. 5. NMDS analysis of (A) bacteria and (B) fungi in the indoor environments of the workshops (70a, 30a, and new). ANOSIM is used to analyze the impact of time and location on (A) bacteria and (B) fungi. All calculations are based on the relative abundance of OTU levels.

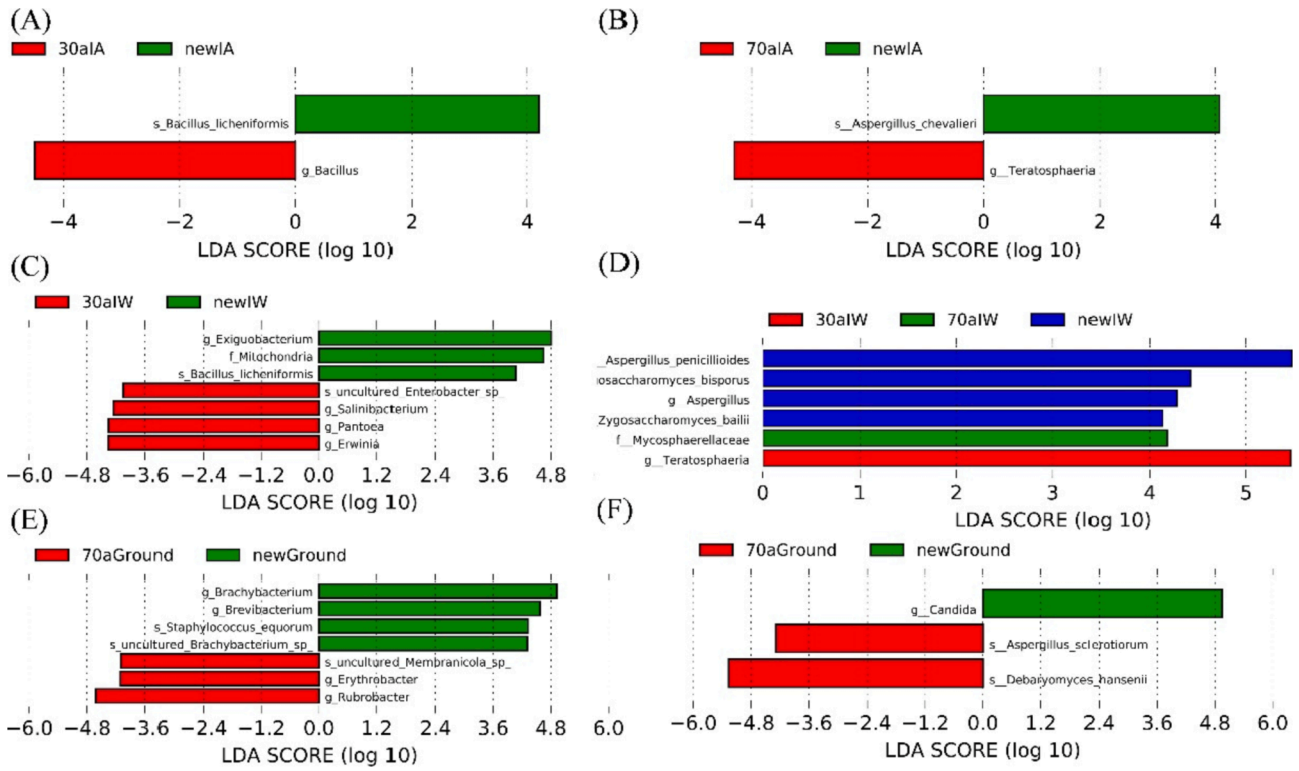

Fig. 6. Linear discriminant effect size analysis of the bacteria and fungi compositions in (A, B) the ground, (C, D) walls, and (E, F) air of the workshop (LDA greater than 4, $p < 0.05$). Histogram of LDA scores calculated for features differentially abundant between groups.

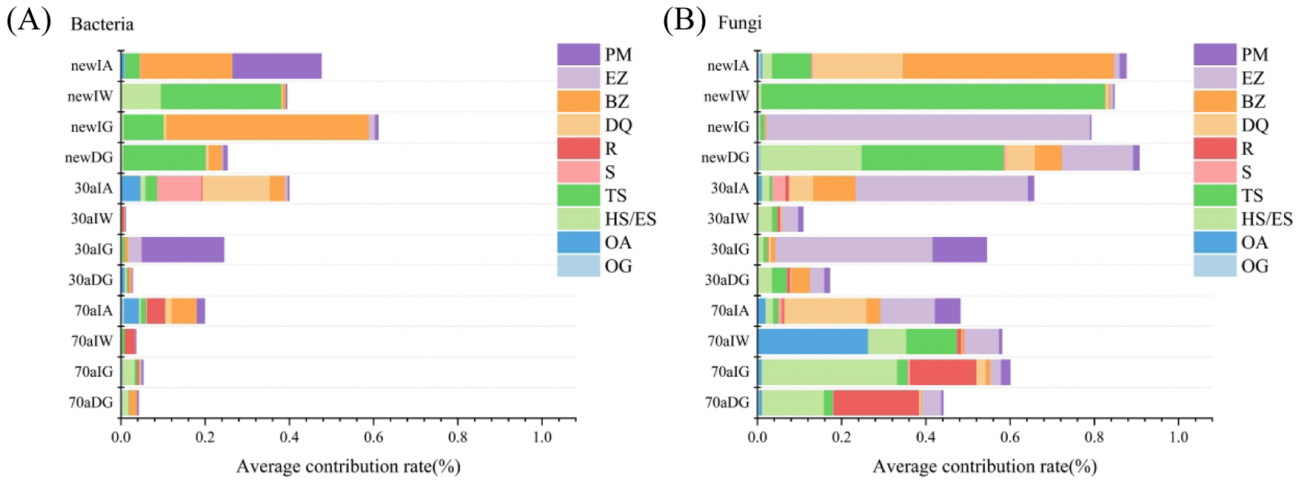

Fig. 7. SourceTracker analysis showing the relative contribution of various sources to the (A) bacterial community and (B) fungal community in the indoor environment of the workshop (PM, pit mud; EZ, fermented Zaopei; BZ, pre-fermentation Zaopei; DQ, Daqu; R, rice husk; S, sorghum; TS, tool surface; HS/ES, worker hand skin surface or equipment surface; OA, outdoor air; OG, outdoor ground).

was the main source of the IG microorganisms. In the new workshop, TS and ES were the important sources of the microbial communities in the indoor environment, especially in IW (contribution of TS, 81.65%); EZ, BZ, and PM were other important sources.

We calculated theβNTI value to compare the influence of the two assembly processes (deterministic and stochastic) of the indoor environmental microbial communities of different usage time workshops (Fig. 8). The results showed that both deterministic and stochastic processes promoted the assembly of environmental microbiome in SFB workshops. For bacteria, as the usage time increased, the contribution of the deterministic process to the community assembly increased, with the proportions of the deterministic process in the indoor environment of different workshops equal to 80.77%, 55.24% and 50.48%, respectively (Fig. 8A). For fungi, the proportions of the deterministic process in the three workshops were not significantly different, at 72.73%, 77.14%, and 69.52% respectively (Fig. 8B).

4. Discussion

SFB is a traditional spontaneously fermented food, and the microbiota from various sources plays a role in fermentation. In this study, for the first time, full-length 16S rRNA/ITS gene sequences were used to comprehensively analyze the microbial community in the SFB brewing environment, and CCS reads of greater than 99% accuracy (Rhoads &

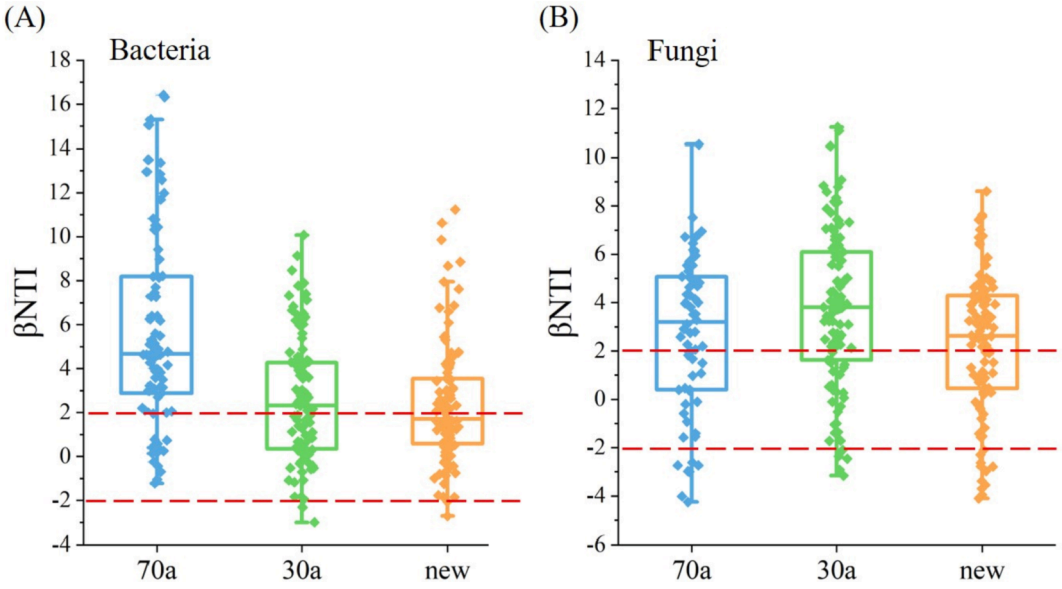

Fig. 8. Distribution box plots of βNTI of (A) bacteria and (B) fungi in the indoor environments of the three workshops. The horizontal red dashed lines indicate the upper and lower significance limits at βNTI = +2 and −2, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Au, 2015) were generated. This yields the potential to improve the accuracy of taxonomic assignments for the known and new species (Pootakham et al., 2017), making the analysis of the diversity and community structure of the microbiota in the SFB brewing environment more accurate.

The prokaryotic community in this study was different from that of previous studies of light-flavor Baijiu in that the environment is the main source of fermentation bacteria (Pang et al., 2018; Wang et al., 2018). Our study showed that the dominant bacterial genera *Lactobacillus* and *Acetilactobacillus* of Zaopei, especially *Lactobacillus acetotolerans*, were not detected in large quantities in the indoor environments of these workshops. In our study, unassigned sequences were quite common at each taxonomic level (for the convenience of presentation, the unassigned part was ignored for display at the genus and species levels), indicating that the SFB brewing workshop have an extremely complex microbial gathering environment that contains a large number of species that cannot be identified in existing databases. The results of the β-diversity analysis (Fig. 4) also showed that the prokaryotic community in the indoor environments of the workshops was more significantly different from that of the other samples with increase in usage time. Baijiu-related functional bacteria were detected in the PM and DQ samples. A previous study showed that DQ was the main source of aerobic bacteria and facultative aerobic bacteria in SFB, whereas PM provided some anaerobes (Wang et al., 2017), which is consistent with our study.

We observed an increase in the prokaryotic diversity of the microbiome in the indoor environment with time of use, especially in the 70a workshop (Fig. S2). The microbial population structure is also an important manifestation of diversity. The environmental community structure of the three workshops presented different structural patterns, each with its own characteristics (Fig. 5). Although there was no significant change in the abundance of the microbiome among the three workshop environments we studied, LEfSe analysis (Fig. 6) of the different microbiomes in the three workshops showed that the composition and abundances of the characteristic microbiomes in the 70a workshop and new workshop were significantly different. Meanwhile, SourceTracker analysis (Fig. 7) revealed that the origin of most of the microbial communities in the 70a workshop environment was "unknown," which may indicate that the long-term brewing process forms a unique microbial community pattern in the environment. βNTI comparative analysis also demonstrated the dominance of the deterministic process of bacterial community construction in the indoor environment of the 70a workshop, indicating that a stable microbiome was constructed in the indoor environment of the workshop under the influence of the long-term brewing process (Xiao et al., 2021). In the new workshop with shorter usage time, BZ, PM, and TS contributed to the bacterial composition of the environment. *B. licheniformis* and *L. acetotolerans* (Pang et al., 2021) were dominant in the Zaopei, TS, IA, and IW samples, and were detected in abundance. This indicates that the functional bacteria of brewing SFB can adapt and transfer to the environment to colonize during the production process, as it transferred to the Zaopei in the subsequent production (De Filippis, Valentino, Alvarez-Ordóñez, Cotter, & Ercolini, 2021).

For the fungal microbial community in the SFB workshop, the brewing functional microorganisms were represented by *S. fibuligera*, *D. hansenii*, *L. ramosa*, *L. corymbifera*, *P. kudriavzevii*, *A. penicillioides*, and *A. chevalieri*, and these species were observed in the environment microecology throughout the SFB brewing process. This demonstrated the adaptation of functional brewing microorganisms to the environment and the potential to affect fermentation as a resident microbiome in these workshops. SourceTracker analysis indicated that Zaopei, PM, and contact surfaces were the fungal sources of the environmental samples, which also supports this view. Owing to differences in microbial colonization, the microbial communities at different locations in the workshop also showed different microbial community structures (Penland et al., 2021). Similar microbial communities occupy the same surface types within each sample type at different locations, reflecting the preferences of the different communities. Comparative analysis of βNTI showed that the fungal community in the SFB workshop environment maintained a high degree of randomness in long-term use, indicating that the fungal community is more susceptible to external influences and changes.

The central feature of traditional fermented food production is the spontaneous fermentation of the raw material by local microbial populations or through colonized processing environments. This process has been used to produce fermented foods for thousands of years. The succession of the microbiome in the environment may be a lengthy process. Currently, the succession process of the microbial community in the Baijiu fermentation workshop is unknown, and there is no research on the long-term tracking of the environmental microbial community. This

study is the first to track the temporal and spatial succession of microbial communities in the SFB brewing environment. As the three workshops were of the same type as the SFB production workshops, a similar technological process allowed us to provide a perspective for the preliminary exploration of the succession of environmental microorganisms in time series.

This was preliminary to the results of our study. We provided a relatively comprehensive microbiome survey of SFB workshops with different usage times. Quality control in the manufacturing environment can be challenging owing to changes in the microbiome, as potential food safety and food quality issues must be considered (Plessas, Alexopoulos, Voidarou, Stavropoulou, & Bezirtzoglou, 2011). Meanwhile, in spontaneous fermentation, represented by Baijiu, controlling the microbiota by, for example, adding microbiomes that are considered to be beneficial to the processing environment, is currently largely impractical (Jin et al., 2017). We believe that our study provides a foundation for research on the microbial communities of the traditional fermented food production environment and systematically promotes the future research direction in this area. Our method can be extended to research involving more complex traditional fermentation environment microbial populations and is also relevant to applications pertaining to improved control of the surrounding conditions in traditional spontaneous fermentation. However, our study has some limitations. It is based on snapshot sampling, which cannot fully capture the dynamic changes between seasons. In addition, many sequences could not be assigned to specific taxonomic groups, which limited our analysis. Nevertheless, in terms of the similarity of microbial communities, we adopted OTU analysis based on a similarity of 97% to improve the reliability of the analysis results. This also suggests that when a better reference database is available, re-examining our analysis may provide a deeper understanding.

5. Conclusions

In summary, this study comprehensively analyzed the microbial distribution and community structure in the SFB fermentation environment at different usage times, using high-throughput full-length sequencing. These findings contribute to a better understanding of the diversity and taxonomic composition of microbial communities in the SFB fermentation environment. Bacterial communities in indoor environments tend to stabilize and develop unique structures during prolonged brewing. The fungal community is more susceptible to a brewing environment. DQ and Zaopei were the main fungal sources, and the community was in a state of dynamic equilibrium during the fermentation process. In view of the importance of the environmental microbiota as a source of brewing microbiota, our work helps to reveal the composition of the microbiome in a spontaneous fermentation environment and further control spontaneous fermentation activity.

CRediT authorship contribution statement

Yilun Li: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – original draft. **Shuangping Liu:** Funding acquisition, Project administration, Writing – review & editing. **Suyi Zhang:** Supervision, Writing – review & editing. **Tiantian Liu:** Supervision, Writing – review & editing. **Hui Qin:** Data curation, Writing – review & editing. **Caihong Shen:** Data curation, Writing – review & editing. **Haipo Liu:** Writing – review & editing. **Feng Yang:** Data curation. **Chen Yang:** Data curation, Writing – review & editing. **Qianqian Yin:** Data curation, Writing – review & editing. **Jian Mao:** Funding acquisition, Supervision, Writing – review & editing.

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.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (32072205,22138004), Sichuan Key Research and Development Project (2021YFS0337), and the first phase of the connotation construction of the 14th Five-Year Plan of Tibetan medicine (2021ZYYGH008).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111126.

References

Abarenkov, K., Henrik Nilsson, R., Larsson, K. H., Alexander, I. J., Eberhardt, U., Erland, S., … Kõljalg, U. (2010). The UNITE database for molecular identification of fungi–recent updates and future perspectives. *New Phytologist, 186*(2), 281–285. https://doi.org/10.1111/j.1469-8137.2009.03160.x

Anderson, M. J. (2010). A new method for non-parametric multivariate analysis of variance. *Austral Ecology, 26*(1), 32–46.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods, 7*(5), 335–336. https://doi.org/10.1038/nmeth.f.303

Coller, E., Cestaro, A., Zanzotti, R., Bertoldi, D., Pindo, M., Larger, S., … Donati, C. (2019). Microbiome of vineyard soils is shaped by geography and management. *Microbiome, 7*(1), 140. https://doi.org/10.1186/s40168-019-0758-7

De Filippis, F., Valentino, V., Alvarez-Ordóñez, A., Cotter, P. D., & Ercolini, D. (2021). Environmental microbiome mapping as a strategy to improve quality and safety in the food industry. *Current Opinion in Food Science, 38*, 168–176. https://doi.org/10.1016/j.cofs.2020.11.012

De Roos, J., Van der Veken, D., & De Vuyst, L. (2019). The Interior Surfaces of Wooden Barrels Are an Additional Microbial Inoculation Source for Lambic Beer Production. *Applied and Environment Microbiology, 85*(1). https://doi.org/10.1128/aem.02226-18

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., … Andersen, G. L. (2006). Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. *Applied and Environmental Microbiology, 72*(7), 5069–5072. https://doi.org/10.1128/AEM.03006-05

Dini-Andreote, F., Stegen, J. C., van Elsas, J. D., & Salles, J. F. (2015). Disentangling mechanisms that mediate the balance between stochastic and deterministic processes in microbial succession. *Proceedings of the National Academy of Sciences of the United States of America, 112*(11), E1326–E1332. https://doi.org/10.1073/pnas.1414261112

Du, H., Wang, X., Zhang, Y., & Xu, Y. (2019). Exploring the impacts of raw materials and environments on the microbiota in Chinese Daqu starter. *International Journal of Food Microbiology, 297*, 32–40. https://doi.org/10.1016/j.ijfoodmicro.2019.02.020

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics, 26*(19), 2460–2461. https://doi.org/10.1093/bioinformatics/btq461

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., & Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics, 27*(16), 2194–2200. https://doi.org/10.1093/bioinformatics/btr381

Edwards, J., Santos-Medellín, C., & Sundaresan, V. (2018). Extraction and 16S rRNA Sequence Analysis of Microbiomes Associated with Rice Roots. *Bio-protocol, 8*(12), e2884. https://doi.org/10.21769/BioProtoc.2884

Fu, J., Chen, L., Yang, S., Li, Y., Jin, L., He, X., … Zou, L. (2021). Metagenome and analysis of metabolic potential of the microbial community in pit mud used for Chinese strong-flavor liquor production. *Food Research International, 143*, Article 110294. https://doi.org/10.1016/j.foodres.2021.110294

Hu, X., Tian, R., Wang, K., Cao, Z., Yan, P., Li, F., … He, P. (2021). The prokaryotic community, physicochemical properties and flavors dynamics and their correlations in fermented grains for Chinese strong-flavor Baijiu production. *Food Research International, 148*, Article 110626. https://doi.org/10.1016/j.foodres.2021.110626

Jin, G., Zhu, Y., & Xu, Y. (2017). Mystery behind Chinese liquor fermentation. *Trends in Food Science & Technology, 63*, 18–28. https://doi.org/10.1016/j.tifs.2017.02.016

Johnson, J., Curtin, C., & Waite-Cusic, J. (2021). The Cheese Production Facility Microbiome Exhibits Temporal and Spatial Variability. *Frontiers in Microbiology, 12*, Article 644828. https://doi.org/10.3389/fmicb.2021.644828

Kembel, S. W., Cowan, P. D., Helmus, M. R., Cornwell, W. K., Morlon, H., Ackerly, D. D., … Webb, C. O. (2010). Picante: R tools for integrating phylogenies and ecology. *Bioinformatics, 26*(11), 1463–1464. https://doi.org/10.1093/bioinformatics/btq166

Knights, D., Kuczynski, J., Charlson, E. S., Zaneveld, J., Mozer, M. C., Collman, R. G., … Kelley, S. T. (2011). Bayesian community-wide culture-independent microbial source tracking. *Nature Methods, 8*(9), 761–763. https://doi.org/10.1038/nmeth.1650

Liu, M.-K., Tang, Y.-M., Guo, X.-J., Zhao, K., Penttinen, P., Tian, X.-H., … Ercolini, D. (2020). Structural and Functional Changes in Prokaryotic Communities in Artificial

Pit Mud during Chinese Baijiu Production. e00829-00819 *mSystems, 5*(2). https://doi.org/10.1128/mSystems.00829-19.

Liu, H., Tan, G., Chen, Q., Dong, W., Chen, P., Cai, K., … Zhao, S. (2021). Detection of viable and total fungal community in zaopei of Chinese strong-flavor baijiu using PMA combined with qPCR and HTS based on ITS2 region. *BMC Microbiology, 21*(1), 274. https://doi.org/10.1186/s12866-021-02334-8

Liu, M.-K., Tang, Y.-M., Guo, X.-J., Zhao, K., Tian, X.-H., Liu, Y., … Zhang, X.-P. (2017). Deep sequencing reveals high bacterial diversity and phylogenetic novelty in pit mud from Luzhou Laojiao cellars for Chinese strong-flavor Baijiu. *Food Research International, 102*, 68–76. https://doi.org/10.1016/j.foodres.2017.09.075

Mao, J., Liu, X., Gao, T., Gu, S., Wu, Y., Zhao, L., … Zhang, J. (2022). Unraveling the correlations between bacterial diversity, physicochemical properties and bacterial community succession during the fermentation of traditional Chinese strong-flavor Daqu. *LWT, 154*, Article 112764. https://doi.org/10.1016/j.lwt.2021.112764

Pang, X.-N., Chen, C., Huang, X.-N., Yan, Y.-Z., Chen, J.-Y., & Han, B.-Z. (2021). Influence of indigenous lactic acid bacteria on the volatile flavor profile of light-flavor Baijiu. *LWT, 147*, Article 111540. https://doi.org/10.1016/j.lwt.2021.111540

Pang, X.-N., Han, B.-Z., Huang, X.-N., Zhang, X., Hou, L.-F., Cao, M., … Chen, J.-Y. (2018). Effect of the environment microbiota on the flavour of light-flavour Baijiu during spontaneous fermentation. *Scientific Reports, 8*(1), 3396. https://doi.org/10.1038/s41598-018-21814-y

Penland, M., Mounier, J., Pawtowski, A., Tréguer, S., Deutsch, S.-M., & Coton, M. (2021). Use of metabarcoding and source tracking to identify desirable or spoilage autochthonous microorganism sources during black olive fermentations. *Food Research International, 144*, Article 110344. https://doi.org/10.1016/j.foodres.2021.110344

Plessas, S., Alexopoulos, A., Voidarou, C., Stavropoulou, E., & Bezirtzoglou, E. (2011). Microbial ecology and quality assurance in food fermentation systems. The case of kefir grains application. *Anaerobe, 17*(6), 483–485. https://doi.org/10.1016/j.anaerobe.2011.03.014

Pootakham, W., Mhuantong, W., Yoocha, T., Putchim, L., Sonthirod, C., Naktang, C., … Tangphatsornruang, S. (2017). High resolution profiling of coral-associated bacterial communities using full-length 16S rRNA sequence data from PacBio SMRT sequencing system. *Scientific Reports, 7*(1), 2774. https://doi.org/10.1038/s41598-017-03139-4

Rhoads, A., & Au, K. F. (2015). PacBio Sequencing and Its Applications. *Genomics Proteomics Bioinformatics, 13*(5), 278–289. https://doi.org/10.1016/j.gpb.2015.08.002

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome Biology, 12*(6), R60. https://doi.org/10.1186/gb-2011-12-6-r60

Tao, Y., Li, J., Rui, J., Xu, Z., Zhou, Y., Hu, X., … Li, X. (2014). Prokaryotic Communities in Pit Mud from Different-Aged Cellars Used for the Production of Chinese Strong-Flavored Liquor. *Applied and Environmental Microbiology, 80*(7), 2254–2260. https://doi.org/10.1128/aem.04070-13

Wang, X., Du, H., & Xu, Y. (2017). Source tracking of prokaryotic communities in fermented grain of Chinese strong-flavor liquor. *International Journal of Food Microbiology, 244*, 27–35. https://doi.org/10.1016/j.ijfoodmicro.2016.12.018

Wang, X., Du, H., Zhang, Y., Xu, Y., & Björkroth, J. (2018). Environmental Microbiota Drives Microbial Succession and Metabolic Profiles during Chinese Liquor Fermentation. *Applied and Environmental Microbiology, 84*(4), e02369–02317. https://doi.org/10.1128/AEM.02369-17

Xiao, C., Yang, Y., Lu, Z. M., Chai, L. J., Zhang, X. J., Wang, S. T., … Xu, Z. H. (2021). Daqu microbiota exhibits species-specific and periodic succession features in Chinese baijiu fermentation process. *Food Microbiology, 98*, Article 103766. https://doi.org/10.1016/j.fm.2021.103766

Zhang, H., Meng, Y., Wang, Y., Zhou, Q., Li, A., Liu, G., … Xing, X. (2020). Prokaryotic communities in multidimensional bottom-pit-mud from old and young pits used for the production of Chinese Strong-Flavor Baijiu. *Food Chemistry, 312*, Article 126084. https://doi.org/10.1016/j.foodchem.2019.126084

Zhang, Y., Shen, Y., Cheng, W., Wang, X., Xue, Y., Chen, X., & Han, B. Z. (2021). Understanding the Shifts of Microbial Community and Metabolite Profile From Wheat to Mature Daqu. *Frontiers in Microbiology, 12*, Article 714726. https://doi.org/10.3389/fmicb.2021.714726

Zhao, J. S., Zhang, S. Y., & Zhou, Z. H. (2009). *The Historic and Cultural Traceability of the Traditional Brewing Technology of Luzhoulaojiao*. Food & Fermentation Technology.

Zheng, Q., Lin, B., Wang, Y., Zhang, Q., He, X., Yang, P., … Huang, X. (2015). Proteomic and high-throughput analysis of protein expression and microbial diversity of microbes from 30- and 300-year pit muds of Chinese Luzhou-flavor liquor. *Food Research International, 75*, 305–314. https://doi.org/10.1016/j.foodres.2015.06.029

Zwirzitz, B., Wetzels, S. U., Dixon, E. D., Stessl, B., Zaiser, A., Rabanser, I., … Selberherr, E. (2020). The sources and transmission routes of microbial populations throughout a meat processing facility. *npj Biofilms and Microbiomes, 6*(1), 26. https://doi.org/10.1038/s41522-020-0136-z