Regulation of the Microbiota in Chinese Liquor Fermentation Process

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Although it is a spontaneous fermentation process of Chinese liquor (*baijiu*) making, the microbial succession keeps surprisingly consistent among batches of fermentation. Three categories of factors are important in driving the microbiota dynamics in liquor making, including the structure of the initial microbiota, the initial environmental factors, and the processing parameters of solid-state fermentation (Fig. 15.1). The liquor-making microbiota can be regulated to control the production of flavour compounds by regulating these factors.

15.1 Regulation of Initial Microbiota in *Baijiu* Fermentation

15.1.1 Sources of the Microbiota

The initial microbiota plays a vital role in driving the microbiota dynamics. Generally, food fermentation is initiated by different natural environmental sources. For example, grapes would provide a complex microbiota for wine fermentation,

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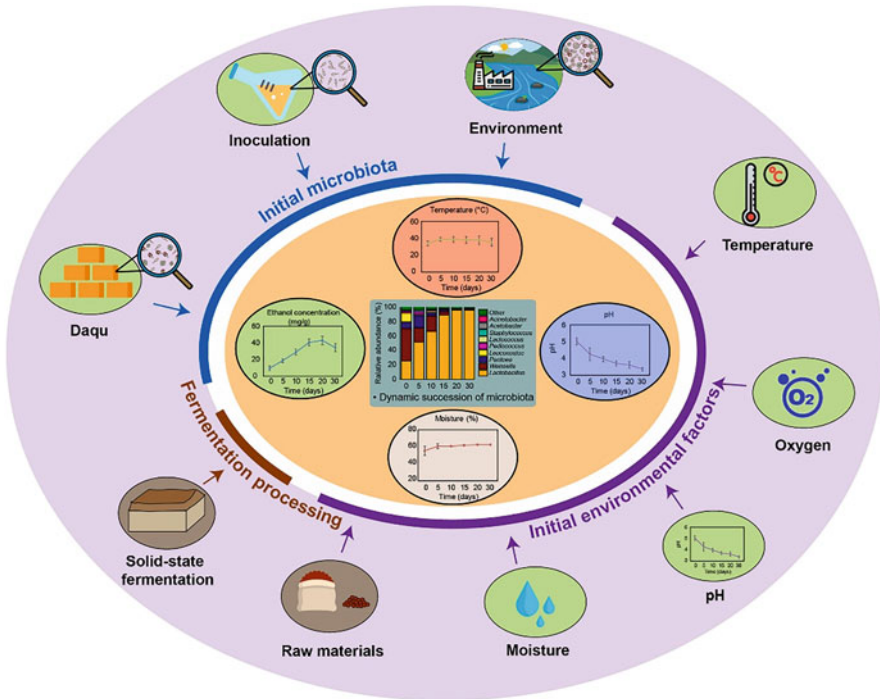
Fig. 15.1 Environmental factors regulating the microbiota in the *baijiu* fermentation process. (Reprinted with permission from [1])

including a number of yeasts and bacteria that is important to wine sensory quality [2]. The different environmental microbiota contributes to food fermentation, and is an important reason why the food quality is different under the same processing technique. There are various environments that can act as microbial sources for *baijiu* fermentation. Figure 15.2 represents the various sources that contribute the microbiota to sauce-aroma *baijiu* fermentation.

15.1.1.1 *Daqu* Fermentation (Starter)

In *daqu* fermentation, the microbiota can be originated from different sources, including raw materials, the air, tools, and indoor ground in the fermentation room. Source tracking analysis of microbiota is performed for medium-temperature *daqu* fermentation. It shows that raw materials contribute 82.67% of bacterial community for new *daqu*, including most of *Pantoea agglomerans*, *Enterobacter aerogenes*, *Pseudomonas koreensis*, and *Sphingomonas desiccabilis*. Tools contribute 55.18% of fungal community for new *daqu*, including most of *Saccharomycopsis fibuligera*, *Rhizopus oryzae*, *Sterigmatomyces elviae*, and *Aspergillus flavus/oryzae*. Raw materials contribute 17.39% of the fungal community, including *Candida athensensis*, *Botrytis cinerea*, and *Sporobolomyces roseus*.
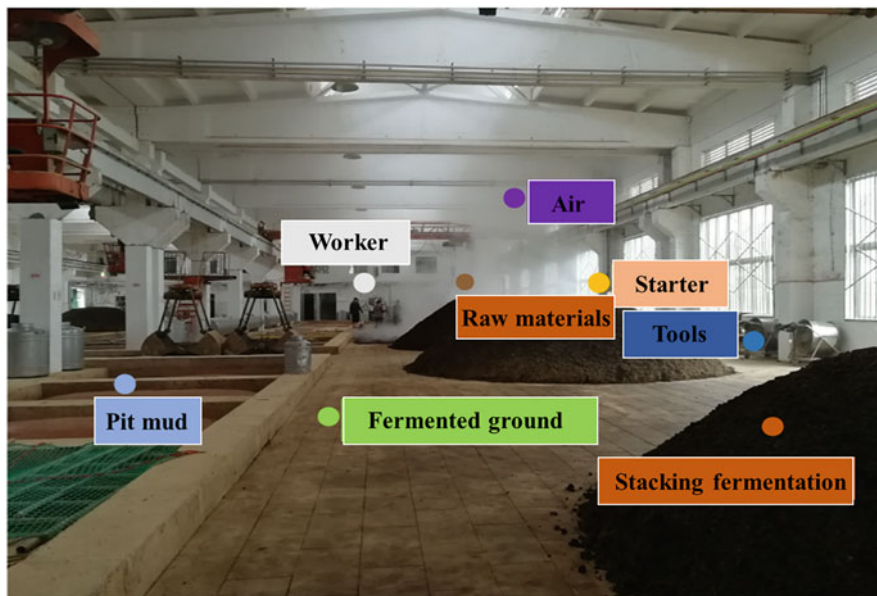
Fig. 15.2 Sources of the microbiota in *baijiu* fermentation. (This picture is kindly provided by Jing Brand Company, Limited)

Indoor ground is also a good contributor, and it contributes 15.97% of the fungal community [3].

15.1.1.2 Stacking Fermentation

Stacking fermentation is a unique processing stage in sauce-aroma *baijiu* making. It is generally recognized as a main stage to accumulate yeast species. The microbiota in different environment is analysed. For bacterial community, *Bacillus*, *Virgibacillus*, *Kroppenstedtia*, and *Oceanobacillus* are dominated in *daqu*. *Lactobacillus* is dominated on the tools and the operating ground. *Pseudomonas* and *Acinetobacter* are dominated in the air. For fungal community, *Thermomyces*, *Thermoascus*, and *Aspergillus* are dominated in *daqu*. *Pichia* are dominated on the tools and the operating ground.

Source tracking analysis reveals that *daqu* provides more than 90% of bacteria for stacking fermentation and about 28% of the fungal community. The operating ground mainly contributes fungi, such as *Pichia* and *Saccharomyces* [4], as shown in Fig. 15.3.

15.1.1.3 *Baijiu* Fermentation

The light-aroma *baijiu* making lacks stacking fermentation, and the fermentation is generally initiated by the natural environment and the starter, *daqu*. In light-aroma *baijiu* fermentation, *daqu* provides with 9.1–27.4% of the bacterial community, and 61.1–80.0% of the fungal community microbiota. The environments, including
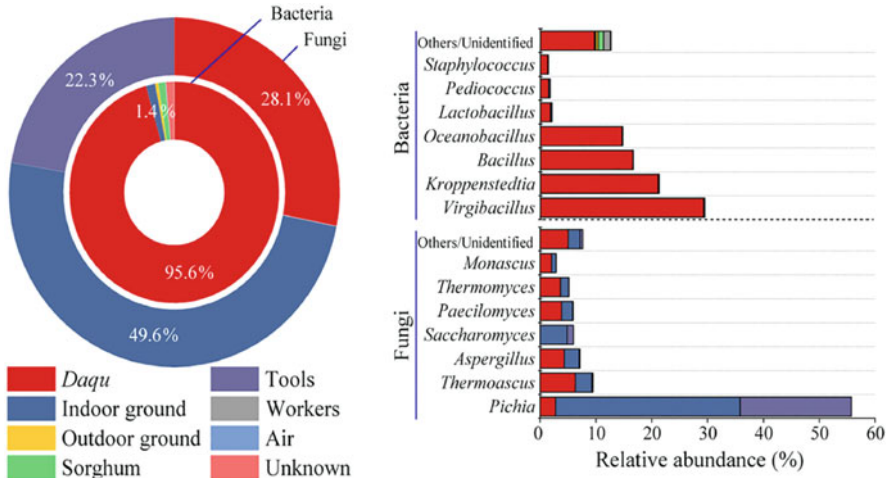
Fig. 15.3 The contribution of environment on bacterial and fungi community to stacking fermentation. (Reprinted with permission from [4])

outdoor ground, indoor ground, tools, etc., provide 62.6–90.9% of the bacterial community and 20.0–38.9% of the fungal community. For example, tools mainly contribute bacterial communities, such as *Lactobacillus*, *Bacillus*, and *Weissella* for one kind of *baijiu* fermentation. Indoor ground mainly contributes fungal communities, such as *Pichia* and *Wickerhamomyces* [5].

The strong-aroma *baijiu* fermentation also lacks stacking fermentation, but contains pit mud in fermentation, therefore, it can be initiated by the microbiota from natural environment, *daqu* and pit mud. Venn analysis reveals that *daqu* mainly contributes strict aerobes and facultative aerobes, over 74% of bacterial community, and pit mud mainly contributes anaerobes (over 14% of bacterial community in *baijiu* fermentation) [6].

15.1.2　Control of the Initial Microbiota

Controlling the initial microbiota is a good way to regulate the fermentation. However, because the initial microbiota can be originated from different sources, the microbiota in the natural environment is difficult to be controlled due to the susceptibility of environmental microbiota to the environmental conditions, such as the geographic and the climate factors. As a result, bioaugmentation might be a good way to regulate the initial microbiota.

15.1.2.1　Bioaugmentation of Microorganisms in *Daqu* Fermentation
For *daqu* fermentation, bioaugmentation of microorganisms is usually applied to regulate the microbiota in *daqu* fermentation. *Bacillus* mainly accumulated in *daqu* plays a major role in flavour compound production, such as pyrazines. It is widely

used to inoculate in *daqu* fermentation to increase the flavour compound production. For example, the inoculation of *Bacillus licheniformis* CGMCC 3963 in *daqu* fermentation efficiently and significantly increases the concentrations of aromatic compounds, and pyrazines, especially the concentrations of pyrazines increased by more than 15-fold. The increase of flavour compounds is not only contributed by the inoculated *B. licheniformis*, but also from the other microbes that might be promoted by *B. licheniformis*. For example, *Bacillus* would promote the growth of *Aspergillus* that contributed to aromatic compounds [7]. In addition, two *Bacillus* species are also inoculated in *daqu* fermentation, including *Bacillus velezensis* and *Bacillus subtilis*. The inoculation increases not only the population of *Bacillus*, but also populations of *Lactobacillus*, and *Candida*. Their inoculation would also increase the enzyme activity related with liquefying, saccharifying, and esterifying activities. In addition, the concentrations of flavour compounds also increase, such as esters, pyrazines, and alcohols [8]. As a result, the bioaugmentation of *Bacillus* would be efficient in improving the quality of *daqu*.

Besides *Bacillus*, a series of microbial mixtures are inoculated in *daqu* fermentation. For example, the combined inoculation of *Bacillus amyloliquefaciens*, *Saccharomycopsis fibuligera*, and *Absidia corymbifera* increases the amylase activity, microbial richness, and contents of several alcohols in *daqu* [9].

Further, the effect of bioaugmented *daqu* on *baijiu* fermentation is also studied. The inoculated *daqu* with *B. velezensis* and *B. subtilis* is used for liquor fermentation with the combination of unbioaugmented *daqu*, and it would increase the concentrations of esters and aromatic compounds in *baijiu* [10]. These findings indicate the bioaugmentation of *daqu* is an efficient way to improve the *daqu* quality, and hence the *baijiu* quality.

15.1.2.2 Bioaugmentation of Microorganisms in *Baijiu* Fermentation

Bioaugmentation is also widely used in *baijiu* fermentation with the aim to increase the *baijiu* quality, including increasing the contents of key flavour compounds, decreasing the off-flavour compounds and the harmful compounds.

As the development of flavour chemistry, the key flavour compounds have been gradually identified. It would promote the development to enhance key flavour compound production via bioaugmentation of microorganisms in *baijiu* fermentation. Esters are one kind of key flavour compounds in *baijiu*. *Wickerhamomyces anomalus* Y3604 is isolated and inoculated in *baijiu* fermentation, leading to the increased concentrations of flavour compounds (mainly ethyl acetate and ethyl caproate), and decreased concentrations of higher alcohols in *baijiu* fermentation [11]. 2-Furfurylthiol is a key flavour compound in Chinese sesame-aroma *baijiu*. L-cysteine is one precursor of 2-furfurylthiol in *baijiu* fermentation. As a result, L-cysteine-high producer could be applied to improve 2-furfurylthiol formation. The two high producers of L-cysteine, *B. subtilis* LBM 10019 and *Bacillus vallismortis* LBM 10020, are isolated in *baijiu* fermentation. The inoculation of these two *Bacillus* strains could efficiently increase the final concentration of L-cysteine (increases by 101.44%) in *baijiu* fermentation, and the concentration of 2-furfurylthiol increases by 89.15% in *baijiu* [12].

Geosmin is an earthy off-flavour compound in *baijiu*. It can be produced by *Streptomyces* spp. The contamination of *Streptomyces* spp. in *daqu* and *baijiu* fermentation would be harmful to *baijiu* quality. *B. subtilis* 2–16 and *B. amyloliquefaciens* 1–45 are isolated in *daqu*. They would significantly inhibit the growth of *Streptomyces* and hence prevent the geosmin production. The application of the indigenous *Bacillus* species is an eco-friendly method to prevent the contamination of *Streptomyces* spp. and geosmin formation in *baijiu* fermentation [13].

Ethyl carbamate (EC) is identified as a group 2A carcinogen. It is necessary to decrease the concentration of EC in food fermentation and fermented foods. Biodegradation is hence used to reduce the concentration of EC in *baijiu* fermentation. Strain *Lysinibacillus sphaericus* MT33, an efficient producer of urethanase and urease, is isolated from *baijiu* fermentation, this strain can degrade 76.52% of EC and 56.48% of urea (the main precursor of EC in *baijiu* fermentation) in simulative fermentation. After inoculation of this strain, the relative abundance of this species significantly increases, and the final EC and urea contents decrease by 41.77% and 28.15% in *baijiu* fermentation, respectively. Consequently, the content of EC decreases by 63.32% in *baijiu* [14].

Cyanide is a main precursor of EC in liquor distillation and storage stages. It can be released from cereal materials in *baijiu* fermentation. As a result, decreasing the concentration of cyanide in fermentation would be important to reduce EC formation in distillation and storage stages. Strain *Saccharomyces cerevisiae* MT-1 is isolated to produce nitrilase, and it can degrade 58.42% cyanide in simulative experiment. The inoculation of this strain could significantly decrease the final concentration of cyanide (by 46.45%) in simulative nitrilase fermentation with additional cyanide [15].

As a result, bioaugmentation has been developed to regulate the flavour compound formation and control harmful compounds in *baijiu* fermentation.

15.2 Environmental Regulation of Microbiota

15.2.1 Regulation of Environmental Factors in *Baijiu* Fermentation at Large Scale

Environmental factors at large scale play important roles in microbial fermentation and final products. A lot of studies reveal the regulation of various environmental factors on microbial community, including pH, temperature, moisture, and salinity. The revealing of microbiota structure and function play important roles for revealing the fermentation characteristics and mechanism for *baijiu* fermentation.

15.2.1.1 Biogeographic Factors Regulating Microbiota in *Baijiu* Fermentation at Spatial Scale

Wine grapes are revealed to present a biogeographic property across viticultural zones that contributes to the quality of wine products. It is revealed that various
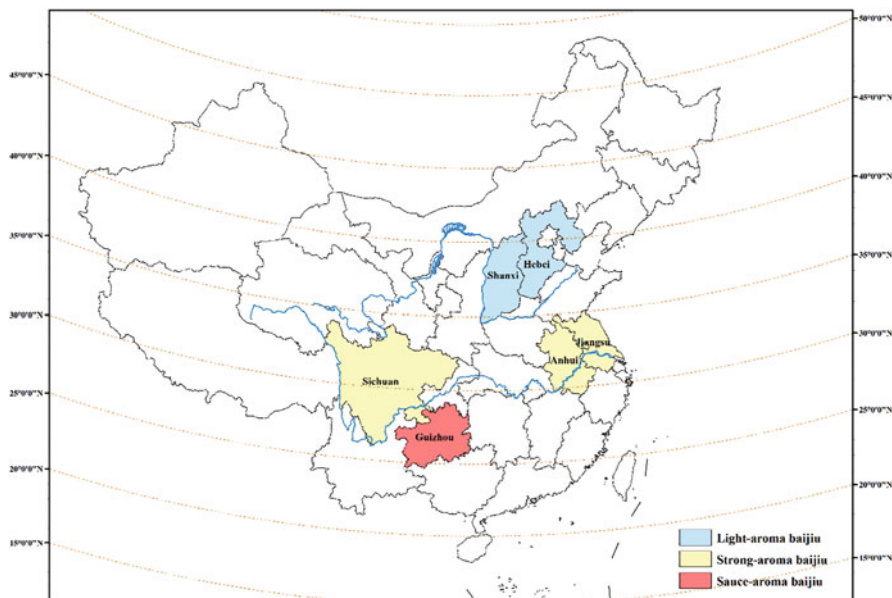
Fig. 15.4 Biogeographic distribution of *baijiu* fermentation

microbes from the environment could inhabit on the surface of wine grapes. The assemblages of the microbiota are correlated with regional, grape variety factors, and climatic features. That is, the environment affects the microbial compositions inhabited on the grape surface, which further contribute to the quality of wine product.

Chinese liquor presents specific geographical distinction and their different aroma types are usually distributed in specific areas (Fig. 15.4). For example, the light-aroma *baijiu* is mainly produced in Shanxi and Hebei provinces; the strong-aroma *baijiu* is mainly produced in Sichuan, Jiangsu, and Anhui provinces; and the sauce-aroma *baijiu* is mainly produced in Guizhou and Sichuan provinces. In addition, the flavour compounds in strong-aroma *baijiu* produced in different areas, such as the Sichuan region and Yangtze-Huaihe River region (or Jianghuai region in Chinese), are also different [16]. The concentrations of 58 flavour compounds are significantly different between *baijiu* from Sichuan and Jianghuai regions. In *baijiu* made in Sichuan region, high contents of pyrazines, furanoids, and carbonyls contribute to the cellar, toasted, and grain aroma, and in *baijiu* made in Jianghuai region, high contents of esters and alcohols contribute to the fruity and floral aroma [17].

The geographical distinction of Chinese liquor might be due to the specific local environments, including local micro-climate and the accumulated microbiota. It is generally accepted that the flavour compounds and aroma of *baijiu* are correlated to their geographical regions.

Zhao et al. studied the relation of regional differences of *baijiu* with the climatic and socio-economic environment, using spatial statistics analysis. Socio-economic factors are positively correlated with the development of the *baijiu* industry, and the climatic factors play an important role in the regional difference of *baijiu*. Among the climate factors, temperature and precipitation would be important for the geographical distribution of *baijiu* [18].

Water is an important part for *baijiu* fermentation as it not only provides the water source and microelements, but also contributes the microbiota for *baijiu* fermentation. The Chishui River originates in Yunnan Province and flows through Sichuan and Guizhou provinces, which is the most famous river that provides water for sauce-aroma *baijiu* making. Moutai liquor is unique owing to the water source from the Chishui River. As a result, the microbial communities in the up- and downstreams of Chishui river in four seasons are studied. It shows that Proteobacteria is the predominant bacterial community (70.16–94.29%), the bacterial OTU peaked in summer. The content of bacteria in the downstream is significantly higher than that in the upstream, and it is slightly higher in spring and summer. For the archaeal community, *Euryarchaeota* and *Thaumarchaeota* are dominant. The species richness of archaeal community is higher in the downstream of all seasons. The differences of bacterial and archaeal community in different streams and seasons of the Chishui River might be related with *baijiu* fermentation in different manufactures. However, the effect of the microbiota from water source on *baijiu* fermentation still need to be revealed [19].

Lactobacillus sp. is important in *baijiu* fermentation. The distribution of this species is studied in *baijiu* fermentation from different locations in China. *Lactobacillus* sp. is widely spread in different *baijiu* fermentations. The distribution of *Lactobacillus* sp. is related with the producing origin, and the abundance of *Lactobacillus* sp. is influenced by the processing way. It indicates that the microorganisms would present biogeographic patterns [20].

15.2.1.2 Climate Factors Regulating Microbiota in *Baijiu* Fermentation at Temporal Scale

The influence of climate on spontaneous food fermentation has been gradually paid more and more attention. Different climate factors driving the microbiota have been identified as important factors. For example, the microbiota in dry and rainy seasons during the manufacturing process of cheese making are revealed. Seasonal factors affect the community structure [21]. In addition, the composition of bacterial community in Chinese cabbage kimchi also presents seasonal difference. The salinity is significantly different with seasons, and it affects the diversity of bacteria, and species *Leuconostoc*, *Lactobacillus*, and *Weissella* present the distribution patterns with seasonality [22]. This work indicates the climate factors would influence the microbiota in food fermentation, and hence lead to different food quality.

Baijiu fermentation is a representative spontaneous fermentation process that is significantly impacted by climate. For example, most strong-aroma *baijiu* makers stop their production by leaving the fermented grains in the pit for more than 3 months at the expense of the production cost in the summer. The quality of

light-aroma *baijiu* produced in summer is worse than those in other seasons. As a result, the regulation of climate factors on microbiota in liquor fermentation is paid more and more attention. Several studies reveal the variation of microbiota in different seasons recently.

Strong-Aroma *Baijiu*

In strong-aroma *baijiu* fermentation, the effect of season is revealed in fermented microbiota. Bacterial microbiota in summer and winter are analysed. Seven phyla are discovered in fermentations of both two seasons. Seven phyla are detected only in the summer fermentation and eight phyla are detected only in the winter fermentation. *Lactobacillus* and *Acetobacter* frequently exist in both winter and summer fermentations. However, the bacterial dynamic variation is different in these two seasons. *Lactobacillus* varies quickly, and becomes the dominant genus at Day 20, and is the absolutely dominant genus at Day 40 (76.3%) in winter fermentation. Whereas, *Acetobacter* accounts for a high abundance in winter fermentation, and is still the most abundant genus at Day 40 (53.1%) in winter samples [23]. This indicates that seasons play important roles on both microbial structure and dynamic variation in strong-aroma *baijiu* fermentation.

Light-Aroma *Baijiu*

The light-aroma *baijiu* can be produced by four seasons with different *baijiu* qualities. Hence, the microbiota and their metabolism in four different seasons are analysed in this aroma type *baijiu* fermentation. For the microbial community, the bacterial OTU number and diversity (indices of Chao 1 and Shannon) are significantly lowest ($p < 0.05$) in summer fermentation, but the fungal diversity is the significantly lowest ($p < 0.05$) in winter fermentation. In addition, that bacteria and fungi show significant differences ($p < 0.05$) in fermentations of the different seasons. The dominant genera are the same, but their relative abundances are different between different seasons fermentations ($p < 0.05$). The difference of microbial community hence leads to a significant difference of the metabolome in different seasons fermentations [24].

Further, indicator microorganisms and metabolites among seasons fermentations are also identified using the random forest analysis. A total of ten genera are identified as seasonal indicator genera, and six metabolites are identified as seasonal indicator metabolites. These seasonal indicator genera are significantly correlated with the indicator metabolites. It is important to figure out the key seasonal driving factors of the microbiota. Among the six seasonal factors (atmospheric pressure, daily average temperature, relative humidity, precipitation, wind speed, and sunshine duration), the daily average temperature has significant ($p < 0.01$) effects on bacteria, fungi, and metabolites, and is identified to be the key seasonal factors driving microbial and metabolite indicators. Hence, an autoregressive moving average model is used to predict seasonal indicators, including ten genera and six metabolites, based on the daily average temperature. Almost all defined indicators are within the limited range of residuals (residuals <0.5) using the prediction model, indicating the seasonal indicators could be predicted by the key seasonal factor [24]
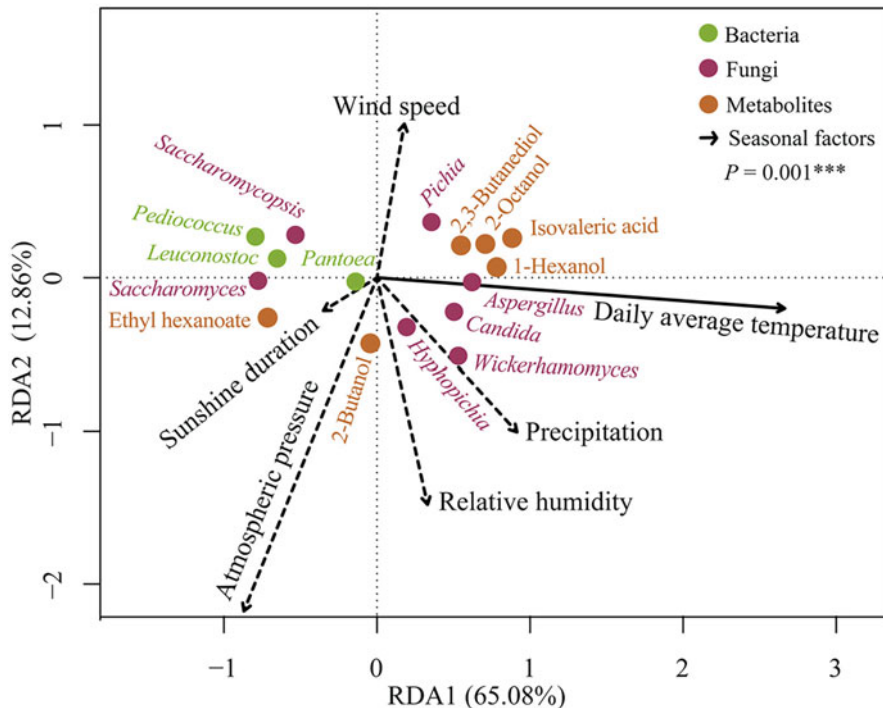
Fig. 15.5 RDA of seasonal factors on microorganisms and metabolites. (Reprinted with permission from [24])

(Fig. 15.5). This work sheds new light on the climate factors on the fermented microbiota and would be used to regulate the microbiota in different seasons fermentations to make stable *baijiu* fermentation.

Although the light-aroma *baijiu* is mainly fermented in vessels embedded in the soil, the daily average temperature would influence the temperature of the soil that is directly in contact with the vessel for *baijiu* fermentation [25]. This would be one mechanism that regulates the microbiota in *baijiu* fermentation. The other mechanism we deduced might be the influence of daily average temperature on the environmental microbiota that initiate *baijiu* fermentation. This mechanism should be further studied.

15.2.2 Regulation of Physicochemical Factors in *Baijiu* Fermentation at Micro-Scale

There are different stages or processes in making of different aroma types *baijiu*. Most aroma type *baijiu*-making processes contain starter and liquor fermentation, such as strong-aroma and light-aroma *baijiu*. But sauce-aroma and sesame-aroma

Fig. 15.6 Schema for sauce-aroma *baijiu* making, including *daqu* making, stacking fermentation, and liquor fermentation. *Daqu* making is characterized by high temperature and low moisture, to enable growth of microbes (mainly filamentous fungi and bacteria)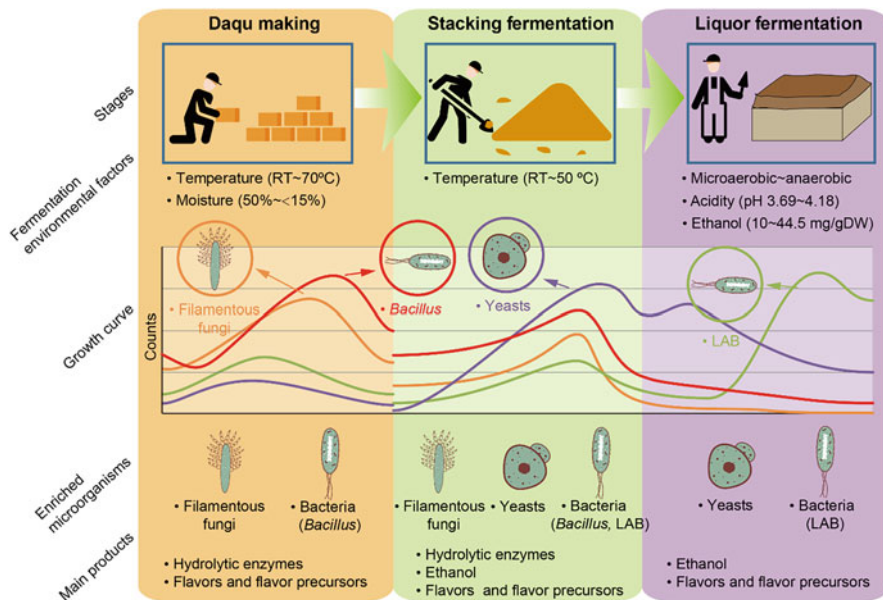 and production of hydrolytic enzymes, flavour compounds and flavour precursors. Stacking fermentation is characterized by high temperature, to enable the growth of microbes (mostly filamentous fungi, yeasts, and bacteria) and their productions of hydrolytic enzymes, as well as a small part of ethanol, flavour compounds, and flavour precursors. Liquor fermentation is characterized by microaerobic to anaerobic fermentation with high acidity and ethanol content. Yeasts enrich in the early period, and lactic acid bacteria (LAB) enrich in the late period. They produce ethanol, flavour compounds, and flavour precursors. *RT* room temperature, *LAB* lactic acid bacteria, *DW* dry weight. (Reprinted with permission from [1])

baijiu making contain three stages, including starter making, stacking and liquor fermentation stages. Figure 15.6 represents a typical sauce-aroma *baijiu* fermentation process.

15.2.2.1 Starter Fermentation

Daqu Fermentation

Daqu, as a starter, provides microbes and other substances, including nutrients, enzymes, flavour compounds and their precursors, for *baijiu* fermentation. The ground wheat (or barley and pea) is mixed with water, and then pressed to form *daqu* bricks. Then, *daqu* bricks are transferred to a room, piled up and covered with straws for spontaneous fermentation. It undergoes 30–40 days of fermentation. During the fermentation, bricks are turned over to control the inner temperature. The covered straw will be removed, the room doors and windows are opened to

exchange the air, remove the moisture and cool the room temperature. The water content of the bricks will gradually decrease, and it is less than 15% at the end of fermentation.

Temperature and water content are important physicochemical factors in *daqu* fermentation. Although bacteria, yeasts, and filamentous fungi grow quickly in the early period, with *Lactobacillales* and *Saccharomycetales* dominating in the initial period [26], the microbial population decreases, and the diversity of fungi also decreases due to the increase of temperature and decrease of water content of *daqu*.

Eighteen filamentous fungal genera are identified in *daqu* by high throughput sequencing analysis. Among them, *Aspergillus* and *Rhizopus* are dominant genera. *Aspergillus* and *Rhizopus* could resist low water activity and high temperature. *Aspergillus* could resist a minimal water activity (a_w) of 0.772 [27] and a maximal temperature of 47.5 °C [28], and *Mucor* could resist a maximal temperature of 57 °C [29]. *Bacillus* genus such as *B. licheniformis* resists a high temperature of 55 °C [30] and *B. amyloliquefaciens* still survives at a_w of 0.917 [31]. As a result, only the thermotolerant and drought-resistant microbes survive, such as *Bacillus*, *Aspergillus*, and *Mucor*, which dominates until the end of *daqu* fermentation [26]. The physicochemical factors play important roles on microbial compositions. The peak temperature affects the diversity of microbial community in *daqu* making. The diversity of bacteria and fungi decrease as the peak temperature increases, and a lowest bacterial diversity is observed in a high-temperature *daqu* by the Shannon index, and the diversity of *Bacilli* reduces dramatically at the end of this stage due to stressed environment conditions [32]. It is suggested the heat resistance of Bacilli is potentially related with the bacterial sporulation, and the spoVA2mob operon, relating with bacterial endospores, might increase the heat resistance of *Bacillus* [33]. As a result, controlling these two factors would regulate the microbiota. If the best dynamic variation is to achieve, it would be beneficial to control the microbiota, and stabilize *daqu* fermentation.

Daqu can be classified to different types based on the maximum fermentation temperature and raw materials, and the different types of *daqu* can be used to make different types of Chinese *baijiu*. For example, high-temperature *daqu* can be used to make sauce-aroma *baijiu*, medium-temperature *daqu* can be used to make strong-aroma *baijiu*, and low-temperature *daqu* can be used to make light-aroma *baijiu*.

High-Temperature *Daqu* for Making Sauce-Aroma *Baijiu*

High temperature plays important roles on flavour compound formation of this kind of *daqu*. *Aspergillus* and *Penicillium* are the most active filamentous fungal genera producing carbohydrate-active enzymes as potential saccharifying enzymes in *daqu*. Moreover, most of enzymes related to the production of flavour compounds are highly expressed in fungal genera, such as *Aspergillus*, *Coccidioides*, *Paracoccidioides*, *Penicillium*, and *Rasamsonia* [34].

High-temperature *daqu* would differentiate to different colours, white, black, yellow, and red *daqu*, due to variations of temperature and moisture. These different coloured *daqu* present different physicochemical characteristics. White *daqu* presents higher protease activity and lower acidity, and black *daqu* presents higher

acidity and lower protease activity. Red *daqu* presents the highest esterification activity, and yellow *daqu* exhibits the highest saccharification and lowest esterification activity. For the microbiota, *Kroppenstedtia*, *Bacillus*, and *Thermoascus* are predominant in all *daqu*; *Saccharopolyspora* and *Thermomyces* are predominant in black *daqu* and red *daqu* [35].

The effect of mechanization on microbial community in *daqu* fermentation is revealed. The bacterial diversity at the end of fermentation in machine-made *daqu* is higher than that in manmade *daqu*, and the dominant genera and their dynamic changes during fermentation present no significant difference. *Rhizobium*, *Bacillus*, *Thermoactinomyces*, *Weissella*, *Lactobacillus*, and *Saccharopolyspora* are dominant in fermentations of both two kinds of *daqu*. The relative abundance of *Bacillus* is higher in the machine-made *daqu* during the fermentation [36].

Medium-Temperature *Daqu* for Making Strong-Aroma *Baijiu*
Various yeast genera such as *Saccharomycopsis*, *Hanseniaspora*, *Geotrichum*, *Wickerhamomyces*, *Yarrowia*, *Candida*, *Alternaria*, *Kazachstania*, *Candida*, *Kazachstania*, *Pichia*, *Saccharomyces*, and *Saturnispora* are observed in medium-temperature *daqu* fermentation, using PCR-denatured gradient gel electrophoresis analysis. Among them, the dominant species are *Pichia kudriavzevii* and *Saturnispora silvae*. In addition, 43 volatile compounds, including esters, acids, alcohols, aldehydes, alkenes, ketone, phenol, furan, and pyrrole, are observed in this kind of *daqu* [37].

Low-Temperature *Daqu* for Making Light-Aroma *Baijiu*
In low-temperature *daqu* fermentation, *Kroppenstedtia*, *Oceanobacillus*, *Lactobacillus*, *Paucisalibacillus*, *Bacillus*, and *Lentibacillus* are the main prokaryotic genera. *Aspergillus*, *Pichia*, and *Saccharomycopsis* are the main eukaryotic microbes. 46 volatile components are identified in this kind of *daqu*, such as esters, alcohols, alkenes, sulphur compounds, ketones, furans, aldehydes, nitrogen-containing compounds, and alkanes [38].

The low-temperature *daqu* can be classified to three types, including Houhuo, Hongxin, and Qingcha *daqu*, based on the difference in temperature during the fermentation. Houhuo presents the highest temperature among the three types of *daqu*, followed by Hongxin, and Qingcha with the lowest temperature. The abundance of fungi is similar, but the abundance of bacteria is different among these three types of *daqu*, based on the counting data. Fungal diversity in Qingcha is higher than that in Houhuo and Hongxin, using PCR-denaturing gradient gel electrophoresis analysis. It indicates that temperature influences the microbial community in *daqu* fermentation. Generally, these three types of *daqu* are all used for making light-aroma *baijiu*. It leads to a high diversity of microbial community to initiate the liquor fermentation, which would be beneficial for the diversity of flavour compounds [39].

Xiaoqu Fermentation
Xiaoqu, as another kind of starter, is smaller than *daqu* by shape. Microbiota in *xiaoqu* fermentation is generally less than those in *daqu* fermentation. Processing is

Fig. 15.7 *Xiaoqu* with brick shapes (left) and with diffused shapes (right). (These pictures are provided by Jiujiang Guangdong distillery Co., Ltd.)

also an important factor regulating the microbiota for *xiaoqu* fermentation. For example, five genera, including *Lactobacillus*, *Aspergillus*, *Pichia*, *Saccharomyces*, and *Rhizopus* are dominant genera by metaproteomics analysis in *xiaoqu* fermentation. Among them, *Lactobacillus*, *Pichia*, and *Rhizopus* are key producers of three glycosidases and two glycosyltransferases, and *Rhizopus* secretes the most abundant glucoamylase. It indicates they are the key saccharifying genera. When the shape of starter changes from the brick to diffused shape (Fig. 15.7), volumetric weight of the starter decreases, the abundance of three genera and the expression of the key saccharifying enzymes significantly decrease. In addition, volumetric weight is positively related with *Rhizopus microsporus*, indicating that volumetric weight is an important driver for the saccharifying microbiota. Further, the relationship between volumetric weight and the physicochemical factors are analysed. The volumetric weight would regulate the moisture and acidity in *xiaoqu*, that would hence regulate the key saccharifying microbiota [40]. As a result, it would be efficient to control the microbiota and the saccharifying enzyme in starter fermentation by regulating the physicochemical factors, via controlling the processing way.

15.2.2.2 Stacking Fermentation

Stacking fermentation is a unique fermentation stage in sauce-aroma and sesame-aroma *baijiu* making (Fig. 15.8). For stacking fermentation, mixtures of mature *daqu* powder and cooled steamed sorghum or/and steamed fermented grains, are piled up on the ground, and ferment for 2–7 days. When the temperature on the top of the stack reaches about 50 °C, the stacking fermentation stops. The time length of

Fig. 15.8 Stacking fermentation

fermentation depends on the ambient temperature, cell growth, and metabolism in this stage.

Temperature is one of the important fermentation environmental factors in this stage. Filamentous fungi multiply in this stage. The total population of filamentous fungi increases and then decreases due to the high temperature in the late period, with a total accumulation fold of 1.7 [41]. *Paecilomyces variotii* and *A. oryzae* are the dominant species [41]. These two species are active hydrolyase producers, with the highest glucoamylase and α-amylase activities. Their populations are consistent with the decreasing contents of starch and the increasing contents of reducing sugar, indicating they might involve in the hydrolysis of starch. In addition, a lot of hydrolytic enzymes accumulate in this stage, consistent with the increase of both saccharifying and liquefying activities. It indicates that the stacking fermentation is not only the stage for multiplication of filamentous fungi, but also for enzyme production, that is important for the simultaneous saccharification and fermentation process in the stacking and the following *baijiu* fermentation stages.

In this stage, bacteria propagates with 1.7- to 5.2-fold on the surface of the stack in different batches [42], with a culture-based method. In addition, it shows significantly different diversity of bacterial community in different batches by PCR-denatured gradient gel electrophoresis technique, and the diversity index in stacking fermentation gradually reduces when the batch number increases [43]. A total of 24 representative species are identified. *Bacillus*, *Staphylococcus*, *Virgibacillus*, *Lactobacillus*, *Thermoactinomycetaceae*, *Streptomyces*, *Lentibacillus*, and some uncultured bacteria are discovered as the dominant bacterial genera in the third to fifth *baijiu* fermentation batches with best *baijiu* quality, indicating these genera might be important for high quality sauce-aroma *baijiu* making. In addition, most of these genera coexist in *daqu*. With Illumina sequencing

method, approximately 26 families of bacteria are identified in this stage, which are the same with those in *daqu* [44]. These indicate that these bacteria in stacking fermentation stage might be originated from *daqu*.

Yeast community variation in a 7-day stacking fermentation stage is also investigated in third liquor fermentation batch. The yeast population increases in the early stage of fermentation, and then decreases in the later period of fermentation. The yeast accumulation fold ranges from about 7.7- to 60.6-fold in different locations of the stack [45], which is much higher than that of bacteria. The maximal temperature on the top reaches 50 °C, and it is aerobic fermentation on the surface of the stack. Although this condition is suitable for both yeast and bacterial growth, yeasts show a better growth trend. This is resulted from the competition between yeasts and bacteria. It is discovered that *S. cerevisiae* inhibits the growth of *B. licheniformis* in their co-culture, even the initial population of *B. licheniformis* is 100 times more than that of *S. cerevisiae*, which explains the better propagation of yeasts in the stacking fermentation [46]. This indicates that the stacking fermentation is an important period for yeast accumulation, but not for bacteria.

Nine yeast species are identified by culture method in this stage. *S. cerevisiae*, *Zygosaccharomyces bailii*, *Pichia membranifaciens*, and *Schizosaccharomyces pombe* are the dominant species [45]. The environmental conditions play important roles on microbial growth. The dynamic population profile and the accumulation fold are different in different positions of the stack. For example, yeast population increases about 60.6-fold on the surface, but it only increases about 7.7-fold in the bottom. In addition, the habitation of yeasts is also different in the stack. *Z. bailii* inhabits in each studied position, *S. cerevisiae* mainly at the top and bottom, *Sc. pombe* at the top and centre, and *P. membranifaciens* mainly on the surface [45]. The different inhabitant location is due to the condition heterogeneity of the stack, such as the temperature profiles and oxygen density. Yeasts play an important role in temperature variation. Since temperature is the indicator of the end of the stacking fermentation based on experience, it indicates the importance of yeasts in this stage, and the obtained population and compositions at the end of stacking fermentation meet the requirement for the following liquor fermentation. As a result, yeast population and structure are essential markers for the stacking fermentation, rather than the temperature.

Recently, using HTS analysis, the diversity of microbial community decreases, and the community structure rapidly succeeds. *Virgibacillus*, *Kroppenstedtia*, *Bacillus*, and *Oceanobacillus* are dominant genera in the early stacking fermentation period, and *Lactobacillus* becomes the dominant genus in the later period. *Pichia* is the dominant fungal genus in the whole stacking fermentation. The quick succession of microbiota increases the accumulation of flavour compounds during the stacking fermentation [4].

15.2.2.3 Liquor Fermentation

After stacking fermentation, the fermented samples are then put into pits or vessels and sealed for fermentation for 30 days or even longer. Oxygen, pH, and ethanol are important fermentation environmental factors in this stage. Due to the consumption

of the limited oxygen and the increasing of ethanol and acid contents, the populations of most of the bacteria, yeasts, and filamentous fungi decrease from the very beginning of the fermentation in pits via a culture-based method [41, 42].

The abundance of filamentous fungi dramatically decreases in *baijiu* fermentation, since filamentous fungi are aerobic microbes. However, the already produced hydrolytic enzymes still exist and activate in fermentation, which is essential for simultaneous saccharification and fermentation process.

Bacterial biodiversity varies significantly during the whole *baijiu*-making process, and it sharply reduces in the fermentation stage in pits. The alpha diversity analysis reveals that the bacterial structure in *baijiu* fermentation becomes much simpler than those of the other two stages, *daqu* and stacking fermentations. It is also quite different from those of the other stages, *Lactobacillaceae* family become the main bacteria in this stage [44], because they are able to resist anaerobic and acidic environments. In this family, the main genus is *Lactobacillus*, and *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus homohiochii*, *Lactobacillus casei*, and *Lactobacillus paracasei* are the dominant species [47].

In addition, productivity and quality of sauce-aroma *baijiu* in different batches are also different. For example, the ethanol content in fifth batch is 2.5-fold than that of seventh batch [48], and the level of esters in fifth batch is also higher than those in seventh batch. The *baijiu* production and yeast population are compared between these two batches. The total yeast population in the fifth batch is two- to fivefold higher than that of the seventh batch in the initial period. *Galactomyces geotrichum* and *S. cerevisiae* are dominant in the fifth batch fermentation, *G. geotrichum* and *S. cerevisiae*, *Issatchenkia orientalis* and *Z. bailii* are dominant in the seventh batch fermentation. *Sc. pombe* is not identified in the seventh fermentation, indicating it might be important species for the high quality *baijiu* of fifth batch [48].

The effects of temperature on bacterial community are also analysed by using different fermented temperature (25, 30, 35, 40, and 45 °C) in simulative *baijiu* fermentation. The relative abundance of bacterial community varies related with the variation of temperature. Temperature significantly influence on bacterial community structure and diversity in the initial 20 days of the fermentation. The diversity of the bacterial community generally increases with temperature. In addition, temperature has a significant influence on ester formation. Low temperature is related with the formation of ethyl acetate, ethyl butyrate, and ethyl caproate, and high temperature is related with the formation of ethyl lactate in the fermentation [49].

15.2.3 Spatial Distribution of Microbial Community in *Baijiu* Fermentation in Pit

Most *baijiu* fermentations are in the pit underground. The microbiota presents spatial distribution in the pit. This would lead to the different characteristics of liquors distilled from the different layers of the pit.

For sauce-aroma *baijiu*, liquors distilled from the upper, middle, and bottom layers of the pit are called top (soy sauce flavour), middle (ethanol-sweet), and

bottom (ester fragrant) *baijiu*, respectively. The ethanol productions in three different layers are different, the maximal level of ethanol is obtained in the upper layer, which is at least twice more than those in the other two layers [45]. The microbiota in three layers are also different. First, ethanol is mainly produced by yeasts, the different ethanol contents are consistent with yeast population in three layers. Using cell count analysis, it is determined that the total population of yeasts is highest in the upper layer among all the three layers, due to the trace amount of oxygen in this layer. In addition, the total population of yeasts keeps stable in the initial 10 days in the upper layer, but the yeast population decreases quickly after Day 5 in the other two layers [45]. *Z. bailii* is determined to be dominant in all the three layers. *S. cerevisiae* and *P. membranifaciens* prefer to inhabit in the upper layer. *Sc. pombe* has the similar population in the middle and bottom layers, and is slightly less than that in the upper layer. The temperatures are also different in the three layers. The temperature of fermented sample is the lowest in the upper layer. It might be due to the cooling of the environment, and the poor heat dissipation, resulting from cell metabolism, leads to the increase in temperature in the other two layers at the first 10 days. But temperatures in the middle and bottom layers decrease gradually after then. The temperature in bottom layer decreases more quickly than that in the middle layer. This is due to lower population and metabolism of microbiota resulted from the complete anaerobic environment in the bottom layer. The difference of temperature would consequently influence the microbial growth and metabolism, and finally leads to the difference of flavour compounds in liquors from three layers [45].

The distribution of bacterial communities in strong-aroma *baijiu* is also analysed in different layers (top, middle, and bottom layers), using phospholipid fatty acid and PCR-denatured gradient gel electrophoresis analysis. Contents of acidity and total esters are the highest in the bottom layer, and ethanol is higher in the top and middle layers. The population of bacteria is the highest in the top layer. Moisture, ethanol, acidity, and reducing sugar are the main factors affecting the distribution of the dominant species, *L. acetotolerans*. The spatial distribution of bacterial communities leads to different quality of *baijiu* from different layers [50].

15.2.4 Regulation of Raw Materials on Microbial Community in *Baijiu* Fermentation

Raw materials play important roles in *baijiu* fermentation. Sorghums are the main raw materials, and other materials are also used for *baijiu* fermentation based on the processing techniques, such as corns, wheats, rice, and glutinous rice. Various kinds of raw materials not only provide different nutrients, but also regulate the microbiota. For example, different combination of raw materials would lead to different successive dynamics of the microbiota. In strong-aroma *baijiu* making, three groups of raw materials are designed, including group S with only sorghum, group T with sorghums, corns and wheats, and group F with sorghums, corns, wheats, rice and glutinous rice as raw materials for *baijiu* fermentation. There are

two stages in the strong-aroma *baijiu* fermentation, according to fermentation parameters. The microbial diversity in the early stage is higher than that in the later stage. Compared with fermentation with single raw material, the microbial succession rate of stage shift is slower in the fermentations with mixed raw materials. In addition, *Lactobacillus* reflects microbial succession rate of stage shift in this type of *baijiu* fermentation. The difference in succession rate of microbial community is able to explain the differences of microbial diversity and flavour compounds. As a result, raw materials would regulate the microbial diversity and flavour compound profile via regulating the microbial succession rate [51].

In addition, the cultivars of the same kind of raw materials could regulate the microbial growth. For example, the effect of sorghum cultivars (glutinous and nonglutinous) on fermentation of *S. cerevisiae* MT and FJ is studied. *S. cerevisiae* MT and FJ are the representative strains for Chinese liquor fermentations respectively with glutinous and nonglutinous sorghums as materials. *S. cerevisiae* MT and FJ exhibit longer stationary phase in fermentations with glutinous and nonglutinous cultivars, respectively [52].

The cultivars of raw materials could also regulate the metabolism of the microbiota in *baijiu* fermentation. For example, hulless barley is the main type of raw material for Qingke *baijiu*. Two different hulless barley cultivars (*heilaoya* and *dulihuang*) are respectively used to make Qingke *baijiu*. The nutrient compositions of these two hulless barley cultivars are different. The content of crude protein is higher in *heilaoya*. However, the content of starch is higher in *dulihuang*. A total of 65 flavour compounds are identified in fermentation. The composition of flavour compounds is similar in fermentations with these two types of hulless barley before Day 10, but differentiated after then. More esters are produced in the late stage of *heilaoya* fermentation, and more carbonyls are produced in the late stage of *dulihuang* fermentation. Six genera (*Lactobacillus*, *Saccharomyces*, *Komagataella*, *Aspergillus*, *Pichia*, and *Weissella*) are correlated with flavour compounds. *Komagataella* dominates in *heilaoya* fermentation and is mainly correlated with esters. *Pichia* dominates in *dulihuang* fermentation and is mainly correlated with carbonyls. Reducing sugar is correlated with the variation of microbiota. Among these sugars, fructose and glucose are significantly correlated with *Komagataella* and *Pichia*, respectively. *Komagataella phaffii* QK2 grows better in fermentation with *heilaoya* containing more fructose, and *Pichia fermentans* PF grows better in fermentation with *dulihuang* containing more glucose in the simulative fermentation. This result highlights that the effect of raw materials on the flavour compound profile is via regulating the compositions of the microbiota [53].

As mentioned above, sugars tend to regulate the structure and metabolism of microbial community in *baijiu* fermentation. However, it is still unclear how the profile of sugars affects the microbial interactions and metabolism. Sugars including maltose, cellobiose, glucose, galactose, and arabinose are observed in *baijiu* fermentation. Among these sugars, glucose and maltose are positively related with the dominant genera *Lactobacillus* and *Saccharomyces* in *baijiu* fermentation. Moreover, a combination of glucose and maltose at a ratio of 9:1 (w/w) enhances the growth of *Lactobacillus* and *Saccharomyces* strains in a simulative *baijiu*

fermentation, and the optimized populations of *Lactobacillus* and *Saccharomyces* strains are 1.21-fold and 1.19-fold, respectively, higher than those with single sugar as substrate. It indicates that combination of specific sugars would promote the microbial growth in *baijiu* fermentation [54].

15.2.5 Regulation of Processing Techniques on Microbial Community in *Baijiu* Fermentation

Recently, mechanization is being paid more and more attention and is gradually used in *baijiu* making. As far as we know, mechanization leads to the decrease of *baijiu* quality. However, the effect of mechanization on microbial community in liquor fermentation is still unknown. As a result, microbial communities in the traditional and mechanized *baijiu*-making processes are studied. The microbial communities are different between fermentations with two processing techniques, and the numbers of yeasts and bacteria at the peak of fermentation by the mechanical technique are significantly higher than those by the traditional technique. Five core microorganisms are identified in the fermentation using metagenomic and metatranscriptomic analyses, including *S. cerevisiae*, *Rhizopus delemar*, *P. kudriavzevii*, *Lactobacillus helveticus*, and *R. oryzae*. The initial abundances of *S. cerevisiae* and *P. kudriavzevii* are higher and the initial abundances of *R. delemar* and *R. oryzae* are lower in the traditional processing technique than that in the mechanized technique. Revealing the effect of mechanization on microbiota in *baijiu* fermentation would be beneficial for processing revolution and making *baijiu* with good quality [55].

15.3 Prospective

15.3.1 Synthetic Microbiota for Contractable and Reproducible *Baijiu* Fermentation

Tradition *baijiu* is produced by spontaneous fermentation with uncontrolled microbiota, synthetic microbiota with core microbiota would be beneficial for contractable and reproducible *baijiu* fermentation. The microbial community reconstruction in cheese rind [56] gives us a reference for the construction of synthetic microbiota of liquor fermentation. Identification of core microbiota is the first step. Different principles are applied to identify the core microbiota. For example, the core microbiota can be identified based on the relative abundances of microbes, microbial contributions to flavour compounds formation, and the stable microbial network. Alternatively, the core microbiota should meet all the requirements, they should be dominant microbiota, flavour-producing microbiota, and co-occurring microbiota. Based on this principle, five genera, *Lactobacillus*, *Saccharomyces*, *Geotrichum*, *Candida*, and *Pichia*, are identified as the core microbiota in *baijiu* fermentation [57]. In addition, the evaluation of the success of synthetic microbiota should also be

considered. The reproducible fermentation can be evaluated based on the dynamic profiles of the microbiota, environmental factors and the flavour compound formation during the fermentation, and the flavour compound compositions at the end of fermentation [57].

The synthetic microbiota is used in natural light-aroma *baijiu* fermentation. It is a rather simple synthetic microbiota, including *R. oryzae* G1 and strains of *S. cerevisiae* Y1 and Y2. The synthetic microbiota is used to substitute the traditional spontaneously fermented starter. Fermentation with the synthetic starter displays lower acidity than that of the traditional starter at the end of fermentation. The microbial dynamics are similar in fermentations with two different starters. *Bacillus*, *Lactobacillus*, and *Lactococcus* are dominant in the fermentation. The relative abundance of *Bacillus* is higher than that of LAB in the early period, but that of LAB is higher than *Bacillus* in the late period [58]. The similarity of microbial dynamics might be resulted from the environmental factors. It indicates that synthetic microbiota should be combined with the control of environmental factors.

15.3.2 Regulation of Environmental Factors

Generally, traditional *baijiu* fermentation is spontaneous fermentation without any control of environmental factors, such as temperature, pH, moisture, and oxygen content. These factors play crucial roles in regulating microbial growth and metabolism, hence alter the outcomes of *baijiu* quality. As a result, it is important to identify the key environmental factors, this would be beneficial for improving and controlling the liquor fermentation via optimizing and controlling these key environmental factors.

Models can be used to simulate and optimize the environmental factors. For example, the environmental factors, such as pH [59], time and temperature [60], can be used to predict the cell growth. In addition, certain biotic factors can also be used to predict the microbial growth in *baijiu* fermentation, such as the initial microbial abundance and microbial interactions [61]. Moreover, models can also be used to predict and optimize the productions of ethanol and flavour compounds [62, 63]. For example, a model is used to simulate the starch conversion and regulate the water loss and the temperature variation in *baijiu* fermentation [25]. As a result, modelling can be an efficient way to predict and optimize the microbial growth and metabolic activities in liquor fermentation.

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