Effect of Solid-state Saccharification Processes on the Flavor of Rice-flavor baijiu

（固体糖化工程が小曲米酒の風味に与える影響）

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Contents

Chapter 1. Introduction

1-1. Baijiu

Baijiu is a world-famous distilled liquor made in China. In 2020, the total consumption of baijiu has reached approximately 7.39 million kL (China Industry Information Network, 2020). The production processes for baijiu are extremely different from those of other distilled spirits such as brandy, whiskey, vodka, gin, and rum (Liu & Sun, 2018). Major differences in production between baijiu and these other distilled spirits encompass the kinds of raw materials used, the fermentation starter, and the fermentation status. Baijiu is usually prepared from raw materials such as sorghum on its own or a mixture of corn, rice, wheat, peas, millet, and sorghum. The exact formulation of these materials affects the flavor of baijiu. In the production of baijiu, jiuqu is a kind of fermentation starter used in China. The grains mixed with jiuqu is simultaneously saccharified and fermented to yield ethanol and various flavor compounds (Liu & Sun, 2018). Jinqu is classified into three types, that is, daqu, xiaoqu, and fuqu. Daqu is a large starter with wheat as the major raw material combined with mold, bacteria and yeast. Xiaoqu, on the other hand, is a smaller starter with rice flour and rice bran as the raw materials, containing mold and yeast. Some kinds of xiaoqu incorporate a small quantity of Chinese herbs. Lastly, fuqu is a dispersed-shape starter with bran as the raw material

combined with pure mold strains. The types of jinqu contribute to the production of daqu-, xiaoqu-, and fuqu-types of liquors (Zhang et al., 2017). Additionally, Chinese liquors are fermented under solid-state, semisolid-state, and liquid state conditions based on their manufacturing techniques. These conditions and the specific periods of fermentation also contribute to make up the flavor.

Currently, it is reported that 12 flavor types of baijiu exist: sauce-flavor, strong-flavor, light-flavor, rice-flavor, miscellaneous-flavor, feng-flavor, dong-flavor, sesame-flavor, te-flavor, chi-flavor, laobaigan-flavor, and fuyu-flavor. Among these flavors, sauce-, strong-, light-, and rice-flavor are the main flavor types of baijiu which exist independently among various aroma types. Another eight flavor types are derived from these four basic aroma types by combining one, two, or more aroma types using their own unique processes (Yu, 2013; Fig. 1-1). In the following section, we will discuss the manufacturing processes and the characteristic flavor compounds in the main flavor types of baijiu.

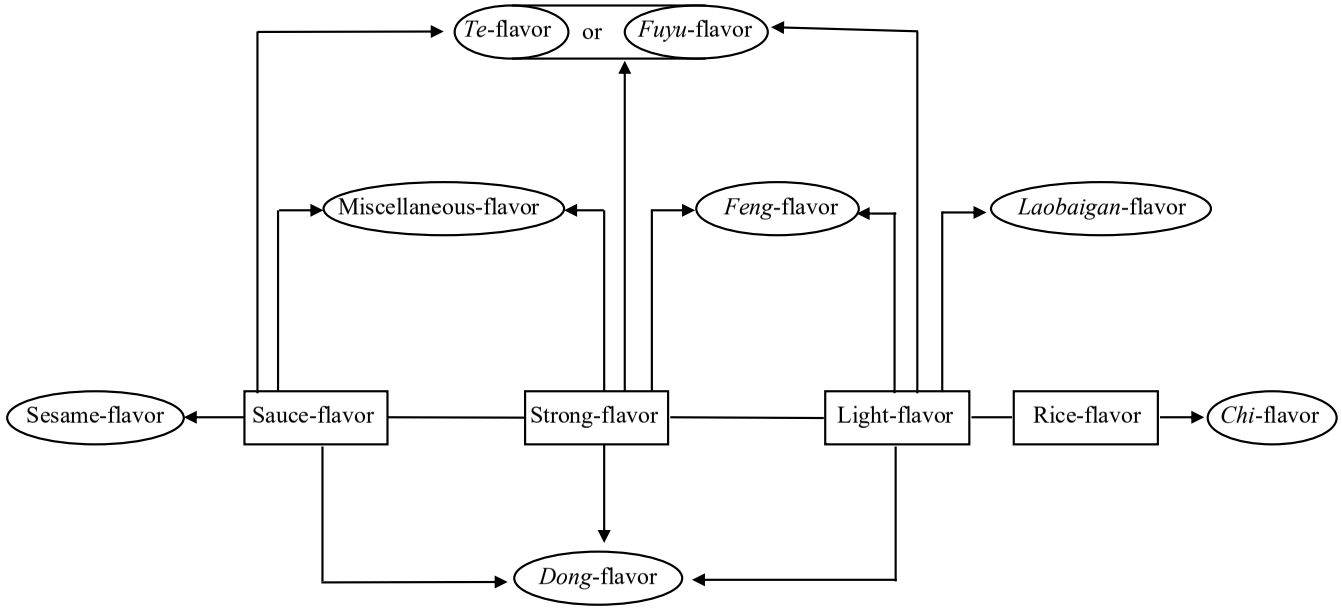

Fig. 1-1. The relationship between 12 flavor types of Baijiu (Yu, 2013).

1-2. The major flavor types of baijiu

1-2-1. Sauce-flavor baijiu

Sauce-flavor baijiu is represented by moutai-jiu, which is known to possess an elegant and delicate aroma with a mellow flavor (Yu, 2013). The production process for sauce-flavor baijiu is depicted in Fig. 1-2. The raw materials used for its production consist of cereals, mostly sorghum, or a mixture of corn, rice, millet, sticky rice, and wheat (Zheng & Han, 2016).

The production process of sauce-flavor baijiu is complex, with a production cycle of 1 year and a storage time of about 5 years. This production process consists of the following steps: (1) First, take 50% of the total raw materials and crush them, and add hot water above 90°C to raise the moisture of the grain to 37%–40%. (2) Then, add 10% of the original zaopei, and steam it for more than 2 hours. Zaopei is a Chinese term for a mixture of fermented grains and microorganisms (Zhang et al., 2012). (3) After the raw material becomes cold, add 10% daqu and 2% tail liquor (poor quality distilled spirits) to mix, stack the zaopei, and ferment it for approximately 5 days. Then, transfer the zaopei to a cellar (mud-bottom and mud-sealed stone pits (Zhang et al., 2017)), and ferment it for 1 month. Remove the zaopei and leave it for a period after fermentation is completed. After crushing the remaining 50% of the raw materials, add hot water above 90°C to raise

the moisture content of the grain to 37%–40%. Thereafter, mix the newly processed raw materials with the zaopei that was previously removed. Then, repeat step (2). (4) Finally, distill the zaopei after fermentation (Wang et al., 2018).

The main representative aroma compounds of sauce-flavor baijiu are phenolic compounds, with a small quantity of amino acids, acids, and esters (Xiong, 2005). Liquor with longer-term storage exhibits the sauce-like flavor more prominently (Fan et al., 2019). Taken together, all the kind of daqu, the fermentation status and period, and storage period contribute to the flavor of sauce-flavor baijiu.

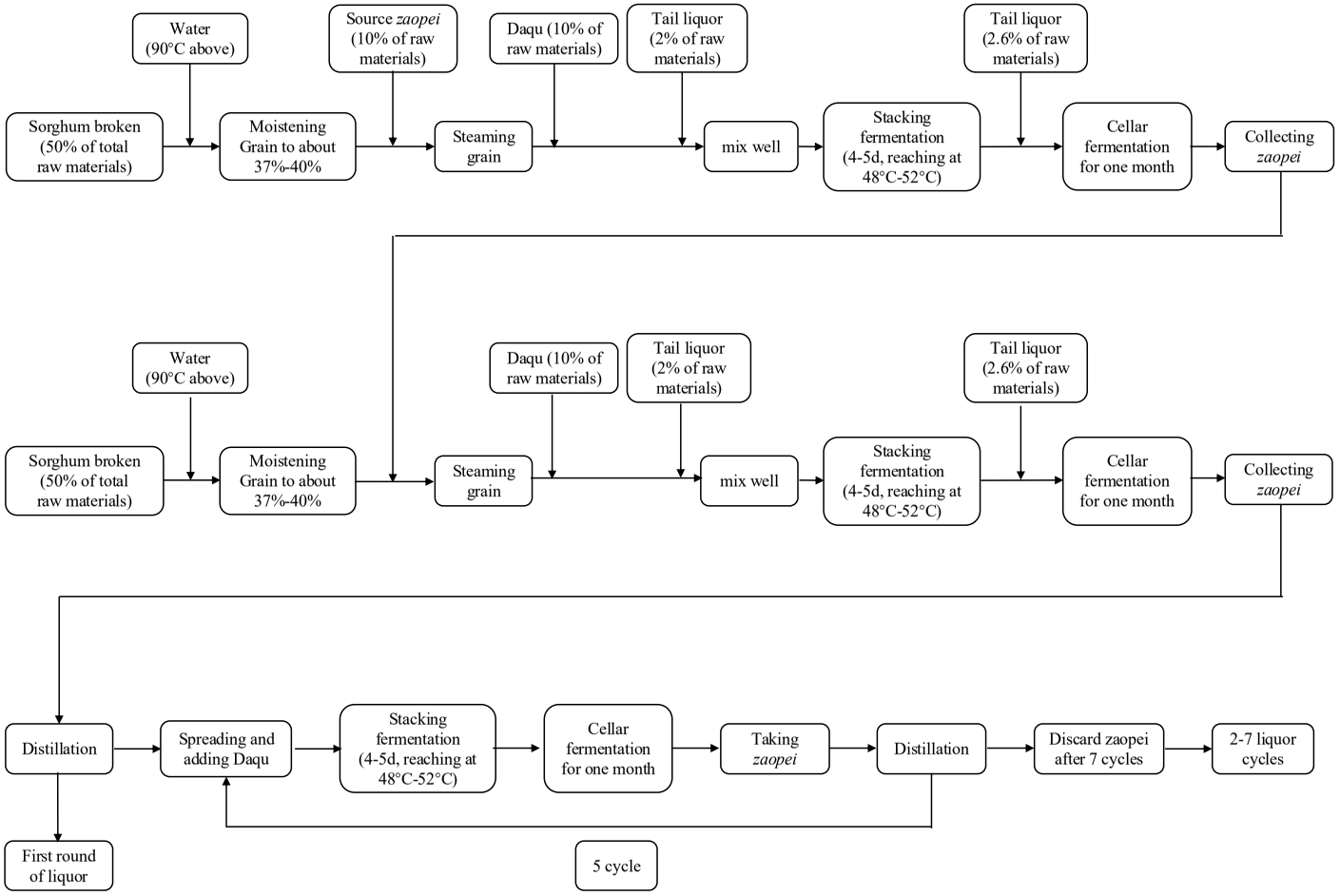

Fig. 1-2. The production method in Sauce-flavor baijiu (Wang et al., 2018).

1-2-2. Strong-flavor baijiu

Strong-flavor baijiu is represented by wuliangye-jiu, which is full-bodied, with a refreshing taste and a lingering aroma after drinking (Yu, 2013). The production process of strong-flavor baijiu is depicted in Fig. 1-3.

The production procedure of strong-flavor baijiu employs the "back-slopping technique" (Zhang et al., 2017). The raw materials for the creation of strong-flavor baijiu are sorghum or corn, rice, millet, glutinous rice, and wheat (Zheng & Han, 2016).

The process for making strong-flavor baijiu is roughly as follows. First, sorghum, rice, and other grains are mixed with zaopei and steamed rice husks. Afterward, the mixture is distilled to yield strong-flavor baijiu. After the distillation, the solid residue is left alone until it is completely cooled; then, daqu powder is added to it and mixed thoroughly. The resulting solid raw material becomes fresh zaopei. Strong-flavor baijiu uses a mud cellar with a volume of about 7 m^3. The mud cellar is covered with yellow mud, which is used to create an anaerobic environment (Jin et al., 2017). The fresh zaopei is then put in the cellar and is left to ferment for 2–3 months (Xu et al., 2010; Zheng & Han, 2016). After the fermentation is completed, the upper layer of zaopei is taken out and distilled to obtain strong-flavor baijiu. The remaining zaopei in the mud pit is used to create another strong-flavor baijiu, by mixing it with grain to enter another cycle (Zou et al., 2018).

Strong-flavor baijiu contains more than 1300 flavor components (Yao et al., 2015).

The representative aroma compounds are predominantly ethyl hexanoate in balance with ethyl lactate, ethyl acetate, and ethyl butanoate (Zheng & Han, 2016). These flavor compounds are produced by the synergistic action of microorganisms in the strong-flavor baijiu ecosystem (Tao et al., 2014). The use of the mud pit and back-slopping technique has been found to be helpful in creating the best composition of the microbial community for the fermentation process and encouraging strong-flavor baijiu to form a strong fragrance (Zheng & Han, 2016).

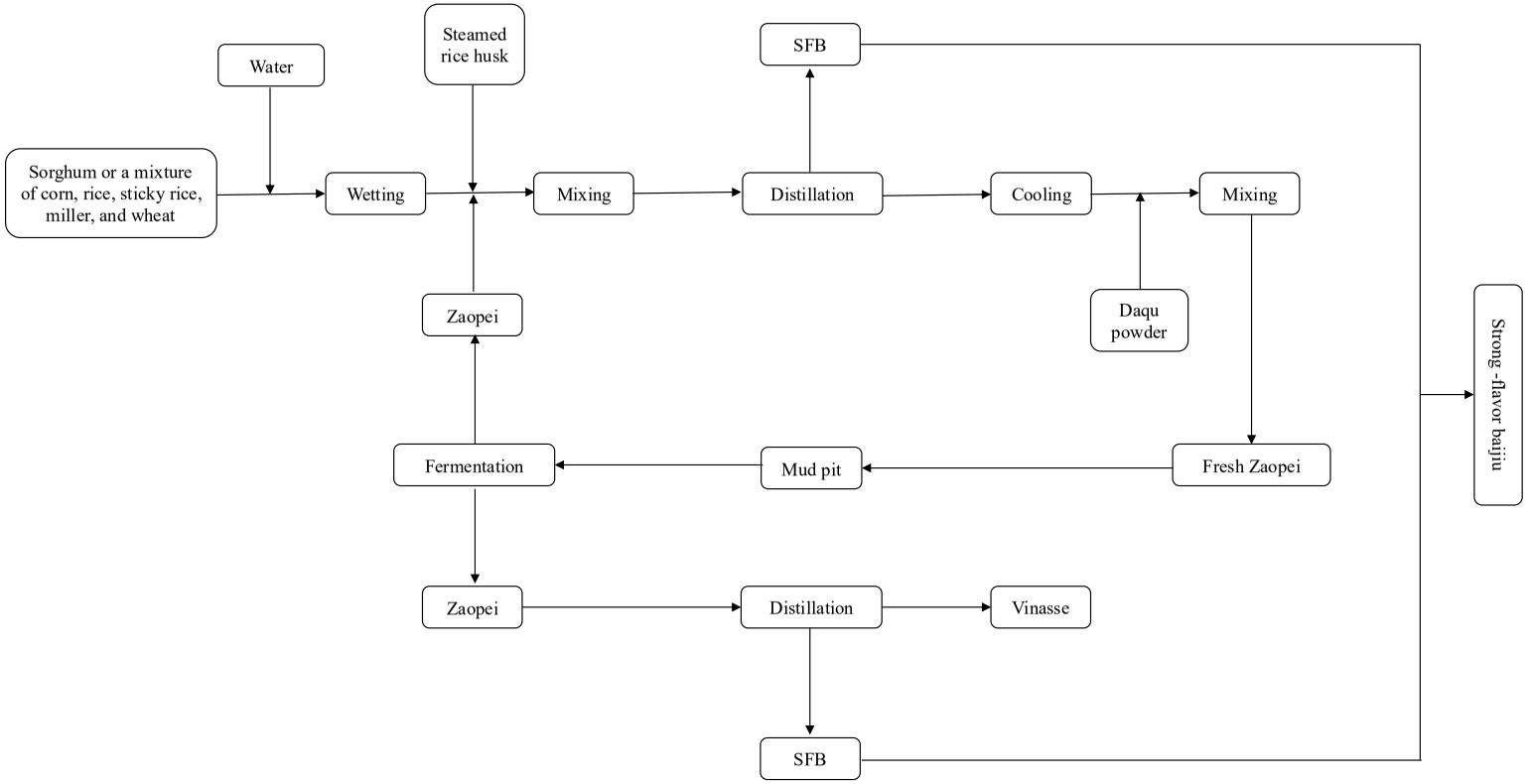

Fig. 1-3. The production method in strong-flavor baijiu production (Zou et al., 2018).

1-2-3. Light-flavor baijiu

Light-flavor baijiu is straightforward to produce and is represented by fen-jiu (Zheng & Han, 2016). Light-flavor baijiu is soft on the palate, with a sweet and refreshing taste, and a fragrance after drinking (Yu, 2013). Fen-jiu is made from sorghum and daqu, and the fermentation occurs in large earthenware containers (Zheng & Han, 2016; Huang et al., 2020).

The production method for light-flavor baijiu is depicted in Fig. 1-4. The main raw material of light-flavor baijiu is sorghum. The first step is the pretreatment of raw materials, which is divided into three main steps: soaking, steaming, and cooling. The sorghum is ground and then soaked in hot water above 85°C for about 20–24 hours to gelatinize the starch. Then, the sorghum is taken out and added to cold water. After cooling, daqu is added and mixed thoroughly with the raw materials (Zheng & Han, 2016; Pang et al., 2020). Next, the pretreated raw materials are placed into an earthen tank to ferment for about 1 month. After the fermentation is completed, the spirit is obtained by distillation. Light-flavor baijiu must be aged for at least 1 year (Zhang, 2003; Zheng & Han, 2016).

The dominant microbes in daqu of light-flavor baijiu are *Lactobacillus* spp., *Weissella* spp., *Pichia* spp., and *Saccharomycopsis* spp. (Hu et al., 2021). During the soaking period of the raw material pretreatment, the microorganisms multiply resulting in the promotion of the acids, esters, and phenols in the wine (Pang et al., 2020). One

study noted that light-flavor liquor had the lowest ethyl carbamate content of 12.02 μg/L among the top th2ree liquor types in China (strong-flavor baijiu is 113.94, sauce-flavor baijiu is 58.10 μg/L). Ethyl carbamate accumulates gradually during the fermentation and storage of liquor and is a genotoxic carcinogen (Zimmerli & Schlatter, 1991; Guan et al., 2021).

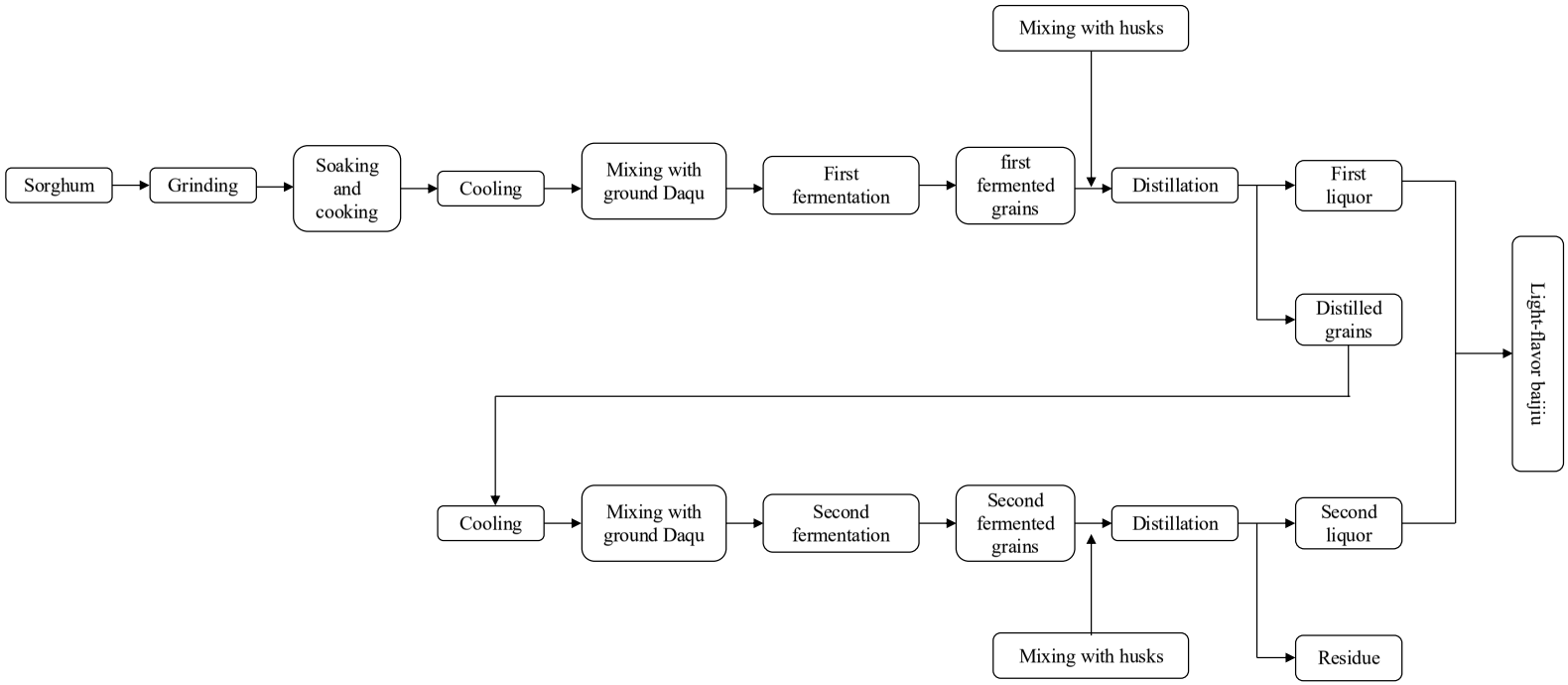

Fig. 1-4. The production method in Light-flavor baijiu (Zheng and Han, 2016).

1-2-4. Rice-flavor baijiu

Rice-flavor baijiu such as guilinsanhua-jiu is made from rice and xiaoqu. The microbial community in xiaoqu consists mainly of yeast, mold, and bacteria (Wu et al., 2017). *Rhizopus oryzae*, which can be found in xiaoqu, is known to play a key role in the brewing of rice-flavor baijiu (Yin et al., 2020). Rice-flavor baijiu's making begins with the inoculation of xiaoqu on steamed rice. The inoculated rice is incubated for approximately 30 h at room temperature. Keeping the mixture's temperature at approximately 35°C. During incubation, *R. oryzae* exhibits vigorous growth and produces enzymes such as α-amylase and glucoamylase and lactic acid (Yin et al., 2020). Approximately 70% of the starch in the rice decomposes to glucose after incubation. This process is referred to as the solid-state saccharification (SSS) because it is incubated in the absence of free-flowing water. After saccharification, water and yeast are added, and the mash is fermented in a liquid state for approximately a week, then the fermented mash is distilled in a liquid-state to obtain a rice-flavor baijiu.

The rice-flavor baijiu is known for its sweet, clean, and refreshing taste in the mouth, and the overall fragrance is elegant (Yu, 2013). Rice-flavor baijiu is produced by using a large stainless steel tank for fermentation and kettle distillation. This type of baijiu is characterized by higher concentrations of *β*-phenylethyl alcohol, ethyl acetate, and ethyl lactate compared to that found in other types of baijiu (Zhang et al., 2017).

1-3. Awamori

Awamori is a kind of shochu which is a traditional Japanese distilled spirit, produced in Okinawa prefecture in Japan. Awamori manufacturing begins with the inoculation of tane-koji on steamed rice and 45 h incubation at 35°C. The proliferation of *Aspergillus luchuensis* from tane-koji during the incubation period produces enzymes required for brewing along with citric acid (Yamada et al., 2011; Zeng et al., 2021). This incubation process is referred to as the “koji-making” process in awamori manufacturing, and the fermented rice is called “koji” in Japanese. Water and yeast are then mixed with koji for alcohol fermentation for about 2 weeks in a liquid state; afterward, the mash is distilled. Awamori is characterized by a higher concentration of 2-heptenal, 1-octen-3-ol, and ethyl myristate than other types of shochu (Fukuda et al., 2016).

1-4. The research purposes

The procedures for making rice-flavor baijiu and awamori are almost the same except for the SSS process and koji making, respectively. Moreover, we note that the primary production areas of rice-flavor baijiu, Guangdong and Guangxi provinces in China, and that of awamori, Okinawa prefecture in Japan, are geographically located next to each other with Taiwan between them. Our previous study revealed several similarities in the manufacturing of both the distilled spirits (Yin et al., 2020). Thus, rice-flavor baijiu and awamori are important distilled spirits for understanding the historical and technical relationships between Chinese and Japanese distilled spirits and their unique development. However, there are few studies of rice-flavor baijiu. Therefore, it is difficult to explicate the relationship between rice-flavor baijiu and awamori manufacturing and flavor.

Thus, in this study, we aimed to investigate the relationships between the manufacturing process and the characteristic flavors in rice-flavor baijiu. First, we analyzed the flavor profile and volatiles present with or without SSS to uncover the effects of the SSS process on the quality of rice-flavor baijiu. Next, we compared the flavor profiles of Chinese rice-flavor baijiu and Japanese awamori—traditional liquors with similar manufacturing processes. Our investigations will contribute to managing the quality of rice-flavor baijiu and to understanding the difference between Chinese and Japanese liquors.

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Chapter 2. Impact of solid-state saccharification (SSS) on the flavor of rice-flavor baijiu

2-1. Introduction

Rice-flavor baijiu is characterized by the light and sweet flavor (Zheng & Han, 2016). It is previously reported that ethyl lactate and acetic acid are key volatile compounds that made it distinct from awamori (Yin et al., 2020b), and β-phenylethyl alcohol is also recognized as the unique compound in rice-flavor baijiu compared to other types of baijiu (Jin et al, 2017). To date, the SSS process has been shown to contribute to the alcoholic fermentation by supplying the required enzymes for brewing (Yin et al., 2020a). However, it has not yet been revealed the effect of SSS on the characteristic volatile compounds of rice-flavor baijiu.

This study aimed to reveal the relationship between the SSS process and the flavor in the rice-flavor baijiu. These pieces of knowledges will contribute to managing the quality of rice-flavor baijiu and understanding the difference between Chinese and Japanese distilled spirits. Thus, we carried out an examination focused on the influence of SSS on the flavor of rice-flavor baijiu by comparing alcoholic fermentation, flavor profiles, and volatile compound levels in rice-flavor baijiu prepared with and without SSS.

2-2. Materials and Methods

2-2-1. Materials, reagents, and strains

All chemicals were acquired from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Nacalai Tesque Inc. (Kyoto, Japan). Xiaoqu (Angel® rice leaven, Angel Yeast Co., Ltd., Hubei, China) was purchased from a local market in China. The yeast strain M6 was isolated from xiaoqu for brewing, which was obtained from the Chinese baijiu industry in Guangdong province (GenBank accession no. LC642156). Polished japonica rice (*Oryza sativa* L.) was purchased from Hombo Shoten Co., Ltd. (Kagoshima, Japan).

2-2-2. Solid-state saccharification (SSS)

A bowl of 450 g of rice was steamed twice to obtain a water content of 60%–63% (Xia, 1989; Shen, 1998; Zheng, 2003). Briefly, the rice was first soaked in water, drained off, and steamed for 1 h, and the required volume of hot water was added until the water content reached 60%. The rice was steamed again for 1 h. After cooling the steamed rice to a temperature of approximately 40°C, xiaoqu (0.45 g = 0.1% of raw rice weight) was mixed thoroughly with the steamed rice. The mixture was incubated at 35°C for 24 h. The

samples were prepared in triplicate and analytical data were obtained from three separate experiments.

2-2-3. Preparation of rice-flavor baijiu with and without SSS

One mash was prepared using the authentic preparation method (Fig. 2-1A) of rice-flavor baijiu in the industry as described below. Steamed rice (450 g of raw rice) was saccharified according to the described process. A total of 475 mL of water and 5 mL of yeast seed culture (2×10^8 cells/mL) were added to the saccharified sample, and the mixture was then incubated at 30°C in a water bath for 7 days for alcoholic fermentation (with SSS). Another mash was prepared by mixing steamed rice, xiaoqu, and water simultaneously without SSS process, and fermented at 30°C for 15 days in a water bath (Fig. 2-1B). The distilled spirit was obtained from single-batch distillation in a glass distillation apparatus (a glass pot coupled with a glass column) in the same manner as that in our previous study (Rahayu et al., 2017). The end of distillation was reached when the alcohol content in the bundled distillate was approximately 38%. The distillate was filtered and diluted with deionized water until the alcohol concentration was 25%. Distilled spirit samples were stored at room temperature in a dark place prior to the analysis. To determine the effect of lactic acid on the fermentation process, lactic acid was added to the mash prepared without saccharification at the same content as that in

the mash prepared with saccharification (SSS) (Fig. 2-1C). The alcoholic fermentation process was conducted at 30°C in a water bath. The samples were prepared in triplicate.

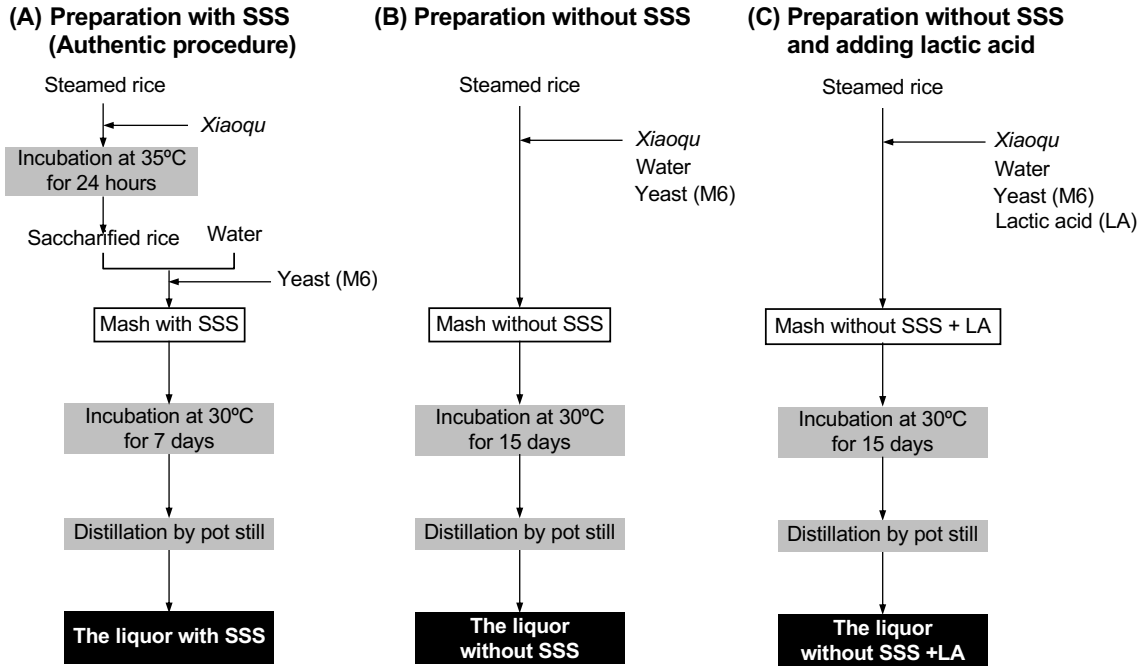

Fig. 2-1. The schematic diagram of rice-flavor baijiu manufacturing used in this study. SSS, solid-state saccharification. (A) The preparation of rice-flavor baijiu with SSS (authentic procedure), (B) the preparation without SSS, and (C) the preparation without SSS and adding lactic acid (LA).

2-2-4. The measurement of yeast cell and survival rate

The cell viability was determined by the methylene blue technique with minor modification (Postgate, 1967). The mash was filtrated by a gauze. A 1 mL filtrate was mixed with a 4 mL deionized water and 5 mL methylene blue solution (0.3 mM in 200 mM glycine buffer, pH 10.2, containing 10% (v/v) ethanol). After thoroughly mixing, the mixture was stand for 15 min. The mixture was resuspended by a vortex mixer, and the total cell and blue-staining cell numbers were counted under microscope. The living cell numbers were estimated by subtracting the numbers of blue-staining cells from total cells numbers.

2-2-5. Monitoring of alcoholic fermentation

Alcoholic fermentation was monitored by measuring the amount of CO_2 gas generated. The samples were weighed after being stirred at the same time each day. The daily decrease in weight from the initial fermentation day was recorded to determine the amount of CO_2 gas generated. The integration curve for weight reduction was plotted on a graph to observe the status of alcoholic fermentation.

2-2-6. Analysis of acetic and lactic acids

Acetic acid and lactic acid were identified using high-performance liquid chromatography (HPLC) (Prominence HPLC system, Shimadzu Corp., Kyoto, Japan) and a conductivity detector (CDD-10A VP, Shimadzu Corp.). The analytic condition was adopted in our previous study (Rahayu et al., 2017). Standard curves were constructed using linear regression of analyte peak areas versus the known concentrations of each compound.

2-2-7. Analysis of amino acids

The concentrations of amino acids were determined using the Prominence HPLC system (Shimadzu Corp.) and an RF-10AXL fluorescence detector (Shimadzu Corp.) by the post-column fluorescence derivatization method. The analytic condition described in our previous study (Rahayu et al., 2017) was employed. Amino acid separation was achieved using an i.d. 6 × 100-mm Shimadzu Shim-pack Amino-Na column (Shimadzu Corp.) at 60°C, and a flow rate of 0.6 mL/min using the amino acid mobile-phase Na-type kit (Shimadzu Corp.). The RF-10AXL fluorescence detector was set to an excitation/emission wavelength pair of 350/450 nm, and the reaction reagents of an amino acid reaction kit (Shimadzu Corp.) were used, maintained at a flow rate of 0.2 mL/min.

2-2-8. Gas chromatography-mass spectrometry (GC-MS) with stir bar sorptive extraction

GC-MS analysis with stir bar sorptive extraction was adopted in our previous study (Yin et al., 2020b). Standard curves were produced from a pure authentic reagent in a 25% (w/w) ethanol solution. The odor active value (OAV) was calculated as the ratio of the concentration of the volatile compounds and its odor threshold.

2-2-9. Sensory evaluation of rice-flavor baijiu

Rice-flavor baijiu with an adjusted alcohol content of 25% were used for the sensory evaluation. Sensory profile analysis was conducted by blind testing by 17 panelists from Kagoshima University (7 males, 10 females; aged 20–57 years). The panelists were previously trained on sensory evaluation techniques for distilled spirit. The panelists evaluated the intensity of each aroma and taste descriptions on a six-point scale (0, not detected; 1, slightly detected; 2, weak intensity; 3, moderate intensity; 4, strong intensity; 5, very strong intensity). Three replications were performed. The results of the sensory profile analysis were averaged for each aroma description and plotted on a spider diagram.

2-2-10. Statistical analysis

Statistical analyses were performed using IBM® SPSS® Statistics version 27 software (IBM, NY, USA). Analysis of variance was performed to compare samples. Significant differences ($P < 0.05$ and $P < 0.01$) between means were determined using the Student's t-test.

2-3. Results and Discussion

2-3-1. Effect of SSS on alcoholic fermentation

To investigate the effect of SSS on alcoholic fermentation, the mash prepared with SSS (mash with SSS) and without SSS (mash without SSS) were compared. Alcoholic fermentation and the growth of yeast were monitored. In addition, lactic and acetic acid concentrations in the mash were measured.

The alcoholic fermentation of the mash with SSS rapidly progresses from day 2 and reached a plateau at 4 days, whereas the alcoholic fermentation in the mash without SSS slowly progressed throughout the fermentation period (Fig. 2-2A). SSS contributes to promote alcoholic fermentation through the initial high glucose content in the mash. However, the alcohol level of the mash without SSS reached the same level at day 14 as that of the mash with SSS. Therefore, it was confirmed that SSS was not necessary for alcoholic fermentation. The growth of the yeast in the mash without SSS rapidly increased at day 2 compared with that in the mash with SSS (Fig. 2-2B). The growth of the yeast in the mash with SSS reached a plateau at day 5, whereas the growth in the mash without SSS gradually and continually progressed throughout the fermentation period. The number of viable cells in the mash with SSS increased until day 3, but decreased as fermentation progressed. The number of viable cells in the mash without SSS

continuously increased until day 9. A slight decrease at the final stage of fermentation was observed. Ethanol is a significant stress factor that interferes with the growth of yeast in the production process of alcoholic beverages (Aguilera & Benítez, 1985). Therefore, it was suggested that the high survival rate of cells in the mash without SSS was a result of the low alcoholic fermentation. Simultaneously, the exponential decrease in the number of viable cells in the mash with SSS was considered to result from the high alcohol content produced by yeast.

The lactic acid concentrations in the mash with SSS were approximately 240-times higher than those in the mash without SSS since the initial stage (Fig. 2-2C). A large amount of lactic acid must be derived from the SSS process (Yin et al., 2020a). In our previous study, we showed that lactic acid bacteria were hardly detected in saccharified rice throughout SSS, and *Rhizopus* sp. grew vigorously during SSS, as revealed by microbial community analysis and cell counts (Yin et al., 2020a). In addition, we confirmed that *Rhizopus* sp. that grew in saccharified rice had lactic acid production ability. Therefore, the difference in lactic acid content in between the mash with SSS and the mash without SSS must result from the lactic acid produced by *Rhizopus* sp. during SSS. Lactic acid concentrations also increased during fermentation. A large amount of lactic acid in the mash with SSS reduced the pH of the mash and repressed the growth of yeast, thereby delaying yeast growth in the mash with SSS on day 2. A large amount of acetic acid was confirmed to be produced in the mash with SSS than in the mash without

SSS (Fig. 2-2C). Acetic acid is one of the key compounds in rice-flavor baijiu and is generally produced by yeast brewed under clean environmental conditions without contamination (Yin et al., 2020b; Nagai et al., 1992). In wine brewing, the production of acetic acid in yeast is influenced by various factors, such as pH or sugar content of the must and the yeast strain (Shimazu & Watanabe, 1981; Caridi et al., 1999; Radler, 1993). When the pH in musts is lower or the sugar content in musts is higher, the formation of acetic acid is promoted by yeast (Shimazu & Watanabe, 1981; Caridi et al., 1999). The initial pH of the mash with SSS was 3.6–3.8, which was lower than the initial pH of 6.4–6.5 of the mash without SSS. The final pH of the mash with SSS was 3.6–3.7. This was almost the same as the initial pH. On the other hand, the final pH of the mash without SSS was 3.9–4.0. Furthermore, the glucose level in the initial mash with SSS was estimated as 20% (w/w). These results indicate that yeasts in the mash with SSS are exposed to low pH and high glucose content for a longer period. Therefore, SSS contributed to the production of a high amount of acetic acid via the low pH and high glucose content in the early stage of fermentation.

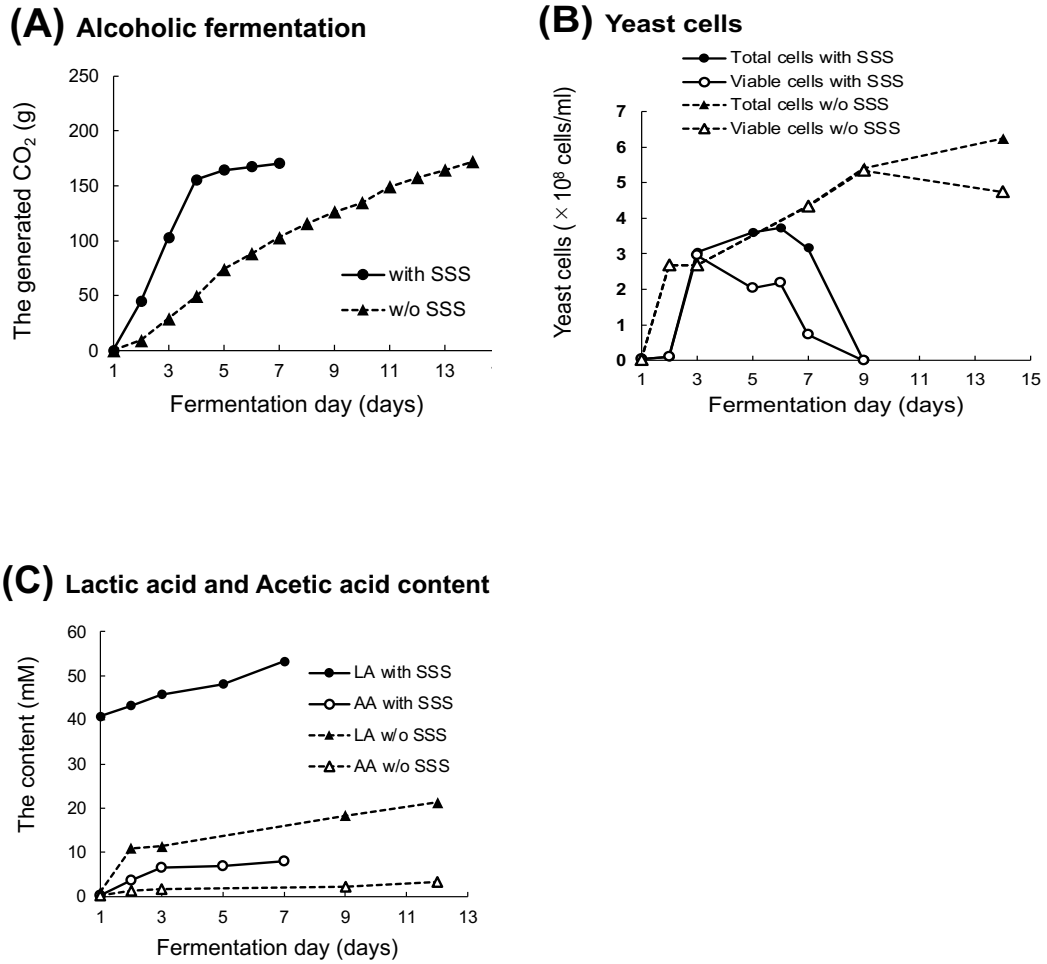

Fig. 2-2. The changes of the alcohol fermentation, yeast cells, and the content of lactic acid (LA) and acetic acid (AA) in the mash. Two kind of the mash was prepared. Each mash was prepared with or without solid-state saccharification process (SSS). Values are represented as the mean of three independent experiments (n = 3). (A) Alcohol fermentation. The mashes prepared with and without solid-state saccharification (SSS) were represented by solid and dotted lines, respectively. (B) The cell number in the mash. The numbers of total cells were represented filled circles and triangles. The number of living cells was represented by open circles and triangles. The mashes prepared with and without SSS were represented by solid and dotted lines, respectively. (C) Lactic acid and acetic acid concentration in the mash. Lactic acid concentrations were represented filled circles and triangles. Acetic acid concentrations were represented by open circles and triangles. The mash prepared with and without SSS was represented by solid and dotted lines, respectively.

2-3-2. Effect of SSS on the flavor of rice-flavor baijiu

The flavor profiles of two rice-flavor baijiu samples were investigated by sensory evaluation and GC-MS. In the sensory evaluation, both distilled spirits exhibited different odor and taste profiles (Fig. 2-3). The distilled spirit prepared with SSS had significantly stronger alcohol-like and floral odors and stimulating mouthfeel (Fig. 2-3A, 2-3B). The distilled spirit prepared without SSS had significantly stronger mold and cheese-like odors. The concentrations of volatile compounds and OAV were determined in the two samples. Twenty-four compounds were identified and quantified: 4 alcohols, 3 acids, 16 esters, and 1 furan (Table 2-1). The distilled spirit prepared with and without SSS contained 13 and 14 compounds, respectively, with an OAV of >1. Among these compounds, the amounts of *β*-phenylethyl alcohol, ethyl lactate, and phenylethyl acetate were significantly larger in the distilled spirit prepared with SSS, while 1-butanol, isoamyl alcohol, butanoic acid, ethyl butyrate, ethyl isovalerate, ethyl caproate, ethyl caprate, isoamyl acetate, and 2-pentyl furan were significantly larger in the distilled spirit prepared without SSS.

Ethyl lactate and *β*-phenylethyl alcohol are the characteristic compounds of rice-flavor baijiu compared with other types of baijiu (Jin et al., 2017). Ethyl lactate was detected only in the distilled spirit prepared with SSS (Table 2-1). Therefore, it was implied that the production of ethyl lactate was correlated with SSS. *β*-Phenylethyl

alcohol and *β*-phenylethyl acetate have rose-like odors. The concentration of *β*-phenylethyl alcohol was significantly higher in the distilled spirit prepared with SSS. The strength of floral odor and the *β*-phenylethyl alcohol concentration in the distilled spirit was consistent. Isobutyl alcohol, isoamyl alcohol, and *β*-phenylethyl alcohol are produced from valine, leucine, and phenylalanine, respectively, by yeast via the Ehrlich pathway or by amino acid synthesis from carbohydrates (Äyräpää, 1965). The concentrations of higher alcohol containing isobutyl alcohol, isoamyl alcohol, and *β*-phenylethyl alcohol are related with the amino acid concentrations in the mash. In studies of distilled spirits prepared from rice (*sake* and *shochu*), many reports showed that lower amino acid contents in the fermented mash promote higher alcohol production, whereas higher amino acid contents in the fermented mash repress higher alcohol production (Setoguchi et al., 2019; Shiraishi et al., 2021; Takamine et al.1989). During the SSS process, the protease activity increases approximately five times (Yin et al., 2020a). This difference in protease activity was maintained throughout the fermentation period for 7 days. Therefore, high protease activity in the mash with SSS might promote high amino acid concentrations and repress higher alcohol production by yeast. Meanwhile, the difference in the concentration of *β*-phenylethyl alcohol was not large between the distilled spirit prepared with SSS and without SSS. Therefore, SSS is not a critical process for the production of *β*-phenylethyl alcohol but contributes to the increase in production by supplying a large amount of phenylalanine to yeast.

Butanoic acid brings unpleasant characteristic odor described as cheese-like for distilled spirit (Wang et al., 2014). The cheese-like odor in the distilled spirit prepared without SSS was derived from butanoic acid (Fig. 2-3A). The amounts of most esters except ethyl lactate and *β*-phenylethyl acetate were higher in the distilled spirit prepared without SSS (Table 2-1). It was previously reported that the concentrations of short-chain fatty acids in the mash affect the corresponding esters (Rahayu et al., 2017). Therefore, it is suspected that the higher concentration of butanoic acid in the mash without SSS facilitated the ethyl butyrate formation. Isoamyl ester of medium-chain fatty acids was also higher in the mash without SSS. It was considered that the higher concentration of isoamyl alcohol in the mash without SSS facilitated the formation of related isoamyl esters. Most esters are responsible for the desired characteristic fruity and floral aroma in alcoholic beverages (Saerens et al., 2010). The distilled spirit prepared without SSS contained a large amount of ester compounds. However, the fruity odor of the distilled spirit prepared without SSS was not significantly different from that of the distilled spirit prepared with SSS (Fig. 2-3). It was considered that the OAV of butanoic acid was too high, and the strong odor masked other odors.

Table 2-1. The concentrations of the volatile compounds in rice-flavor baijiu.

Name		RI	Identification method	Quantification ion	The concentration (μg/L) with SSS	The concentration (μg/L) w/o SSS	Odor threshold (μg/L)	OAV with SSS	OAV w/o SSS	Ref
(Alcohol)										
Isobutyl alcohol	Fusel, alcohol	1,063	MS, RI, STD	43	1,208,741 ± 126,975	1,113,574 ± 94,938	40,000	30	28	1
1-Butanol	Medicinal, alcohol	1,113	MS, RI, STD	56	3,541 ± 389	10,755 ± 1,122	5,000	<1**	2.2	2
Isoamyl alcohol	Alcohol, harsh, bitter	1,171	MS, RI, STD	55	469,252 ± 76,086	681,974 ± 47,931	30,000	16**	23	1
β-Phenylethyl alcohol	Floral, alcohol	1,830	MS, RI, STD	91	52,485 ± 1,917	44,564 ± 2,326	10,000	5**	4	1
(Acid)										
Butanoic acid	Cheese, unpleasant	-	MS, RI, STD		N.D.	71,458 ± 57,694	173	-	413	3
Decanoic acid	Fatty, unpleasant	2,181	MS, RI, STD	60	2,263 ± 60	2,408 ± 164	15,000	<1	<1	1
Dodecanoic acid	Dry, metallic, laurel oil	2,385	MS, RI, STD	73	1,078 ± 42	1,045 ± 34	15,000	<1	<1	2
(Esters)										
Ethyl butyrate	Fruity, pineapple	986	MS, RI, STD	71	320 ± 29	13,613 ± 3,584	20	16**	681	1
Ethyl lactate	Fruity, lactic, raspberry	1,292	MS, RI, STD	45	35,799 ± 4,352	N.D.	14,000	2.6**	-	2
Ethyl isovalerate	Fruity	1,472	MS, RI, STD	88	5.9 ± 2.9	30 ± 2	3	2**	10	1
Ethyl caproate	Fruity, floral	1,193	MS, RI, STD	88	678 ± 40	809 ± 67	5	136*	162	1
Ethyl caprylate	Pineapple, peer, floral	1,398	MS, RI, STD	88	3,737 ± 189	4,137 ± 385	2	1869	2068	1
Ethyl caprate	Fruity, fatty, solvent	1,597	MS, RI, STD	88	1,533 ± 151	3,475 ± 1,109	200	7.7*	17.4	3
Ethyl laurate	Sweet, floral, fruity	1,783	MS, RI, STD	88	42 ± 12	71 ± 32	500	<1	<1	4
Ethyl myristate	-	1,987	MS, RI, STD	88	47 ± 8	36 ± 12	500	<1	<1	4
Ethyl oleate	-	2,448†	MS, RI, STD	264	17 ± 6	25 ± 9	870	<1	<1	2
Ethyl linoleate	-	2,441†	MS, RI, STD	67	878 ± 190	727 ± 163	450	2.0	1.6	2
β-Phenylethyl acetate	Floral, rose	1,743	MS, RI, STD	104	593 ± 22	388 ± 26	250	2.4**	1.6	1
β-Phenylethyl butyrate	Fruity	1,886	MS, RI, STD	104	1.18 ± 0.01	22 ± 22	961	<1	<1	5
Isoamyl acetate	Banana	1,083	MS, RI, STD	70	1,663 ± 159	4,481 ± 730	30	55**	149	1
Isoamyl hexanoate	Pineapple, cheese	1,413	MS, RI, STD	70	0.33 ± 0.02	1.2 ± 0.1	1,400	<1**	<1	2
Isoamyl octanoate	Sweet, light, fruity, cheese	1,609	MS, RI, STD	70	3.8 ± 0.3	15 ± 2	125	<1**	<1	3
Isobutyl octanoate	-	1,501	MS, RI, STD	127	1.5 ± 0.1	2.0 ± 0.2	800	<1**	<1	6
(Furan compound)										
2-Pentylfuran	Green bean-like	1,208	MS, RI, STD	81	2.8 ± 0.2	4.0 ± 0.6	1	2.8*	4.0	7

Data are means ± SD (n=3). Method of identification: MS, mass spectrum comparison using NIST05a library; RI, retention index in agreement with literature value; STD, confirmed by authentic standards. †These RIs were estimated by adapting the conversion formula from retention time to RI prepared in this study as RI was slightly out of the range. N.D., not detected.

Odor threshold obtained from references: [1] Guth, 1997; odor threshold in 10% (w/w) ethanol, [2] Salo et al., 1972; odor threshold in synthetic wine (11% (w/w) ethanol), [3] Ferreira et al., 2000; odor threshold in synthetic wine (11% (w/w) ethanol), [4] Zea et al., 2001, [5] Wang et al., 2014; odor threshold in synthetic Chinese liquor (46% (w/w) ethanol, [6] Li et al., 2008: odor threshold in 12% (w/w) ethanol containing 5g/L tartaric acid at pH 3.2, [7] Buttery et al., 1988; odor threshold in water. Significant difference (*$P < 0.05$, $P < 0.01$**) between means were determined using Student's *t*-test.

Table 2-2. Amino acid concentrations in the mash .

	with SSS	w/o SSS	
Phenylalanine	53 ± 1	39 ± 2	**
Threonine	21 ± 4	17 ± 4	
Isoleucine	6 ± 2	7 ± 2	
Leucine	26 ± 5	19 ± 1	**
Valine	13 ± 3	11 ± 1	
Total	553 ± 54	454 ± 111	

Data are mean ± SD. Significant difference (**$P < 0.01$) between means were determined using Student's t-test.

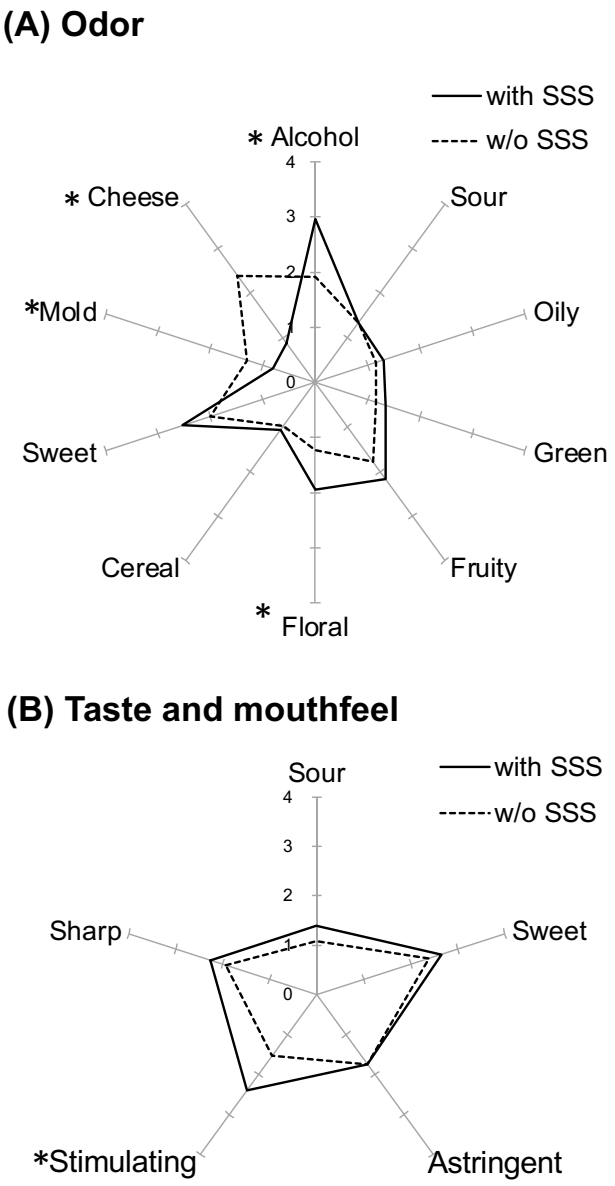

Fig. 2-3. The comparison of the odor and taste profiles of rice-flavor baijiu with or without solid-state saccharification. The rice-flavor baijiu with or without solid-state saccharification (SSS) were indicated by solid and dotted lines, respectively. The sensory evaluation was carried out by 18 panelists. The panelist evaluated the intensity of the odor and taste in the following manner: 0 = not detected, 1 = slightly detected, 2 = weakly detected, 3 = detected, 4 = strongly detected, and 5 = very strongly detected. The values are shown as the means. Significant difference (*P < 0.05) between means were determined using Student's t-test.

2-3-3. Effect of lactic acid in the mash

Because the lactic acid concentration in the mash with SSS was very high throughout the alcoholic fermentation period (Fig. 2-2), it was assumed that the increased lactic acid concentration induced the elevated ethyl lactate concentration in the distilled spirit. In addition, butanoic acid was not detected in the distilled spirit prepared with SSS. Butanoic acid is converted from lipids by lactic acid bacteria such as *Lactobacillus plantarum* (Azarnia et al., 2006). Therefore, it was suggested that lactic acid produced by *Rhizopus* sp. maintains the low pH of the mash and contributes to prevent the production of butanoic acid by bacteria. To determine the effect of lactic acid in brewing, the mash without SSS was prepared by adding the lactic acid equivalent to the mash with SSS (without SSS+LA), and the mashes was prepared with or without SSS were compared (Fig. 2-1C). The lactic acid, acetic acid, butanoic acid, and ethyl lactate concentrations in the mash at the beginning and final stages of fermentation and the distilled spirit were analyzed.

The lactic acid concentration during fermentation was increased in the mash without SSS (Table 2-3). Although ethyl lactate was not detected in the early stage of alcoholic fermentation in all mashes, it was detected in the final stage in the mash without SSS+LA and the mash with SSS. Hara et al. (1968) showed that the amount of ethyl lactate was larger than that of lactic acid in *sake* mash. This report is correspondent with

our results. We found that the esterification of lactic acid and ethanol by yeast does not proceed rapidly. Thus, SSS facilitates the production of ethyl lactate by increasing the concentration of lactic acid in the mash since the initial fermentation.

Acetic acid was also detected higher in the mash without SSS+LA and the mash with SSS at the early fermentation stage (Table 2-3). The acetic acid concentration was higher in the distilled spirits prepared without SSS+LA and with SSS than tin those prepared without SSS. This finding confirmed our hypothesis that SSS contributes to the high production of acetic acid via the decrease in mash pH. In other words, SSS is not critical process to produce acetic acid unlike *β*-phenylethyl alcohol, but it contributes to the increase in acetic acid production by yeast. In contrast to acetic acid, butanoic acid was not detected in the mash without SSS+LA and with SSS. Therefore, lactic acid at the initial fermentation stage must play a role in decreasing the production of butanoic acid by preventing the proliferation of lactic acid bacteria.

In this study, we demonstrated that SSS of rice-flavor baijiu contributes not only to brewing but also to the production of the characteristic flavor compounds, *β*-phenylethyl alcohol, ethyl lactate, and acetic acid in rice-flavor baijiu, and this is an important process to repress the off-flavor derived from contamination.

Table 2-3. The concentrations of lactic acid, ethyl lactate, acetic acid, and butanoic acid in the mash and liquors

		Mash		
		Day 2	Final day*	Liquor
Lactic acid	w/o SSS + LA	32.76 ± 1.57	51.98 ± 2.10	ND
(mM)	with SSS	47.51 ± 0.72	52.51 ± 0.89	ND
	w/o SSS	7.03 ± 0.60	27.04 ± 1.74	ND
Ethyl Lactate	w/o SSS + LA	0.022 ± 0.038	0.61 ± 0.28	0.96 ± 0.30
(mM)	with SSS	0.076 ± 0.002	0.46 ± 0.01	0.30 ± 0.03
	w/o SSS	ND	ND	ND
Acetic acid	w/o SSS + LA	0.90 ± 0.05	11.06 ± 0.08	2.96 ± 0.57
(mM)	with SSS	1.87 ± 0.27	9.56 ± 0.27	1.93 ± 0.17
	w/o SSS	0.52 ± 0.02	4.01 ± 0.63	0.56 ± 0.18
Butanoic acid	w/o SSS + LA	ND	ND	ND
(mM)	with SSS	ND	ND	ND
	w/o SSS	ND	0.12 ± 0.01	0.04 ± 0.04

*The 'last day' of sample w/o SSS + LA is day 15.

*The 'last day' of sample with SSS is day 7.

*The 'last day' of sample w/o SSS is day 15.

Data are means ± SD (n=3). ND means not detected.

2-4. Summary

To date, the application of SSS of rice-flavor baijiu making is recognized only for the saccharification to brew the distilled spirit. This study showed that SSS plays an important role in producing the characteristic flavor compounds of rice-flavor-baijiu; *β*-phenylethyl alcohol, ethyl lactate, and acetic acid. The production of *β*-phenylethyl alcohol by yeast is facilitated by the high concentration of phenylalanine in the mash provided by the decomposition of proteins during SSS. SSS produces large amounts of lactic acid. This high lactic acid concentration in the initial fermented mash promotes ethyl lactate synthesis by yeast. In addition, the high lactic acid concentration in the mash induces the decrease in mash pH, and the low pH of the mash promotes acetic acid production by yeast. Moreover, butanoic acid production is also repressed by the high concentration of lactic acid in the mash since the initial fermentation stage. We demonstrated that SSS contributes to repress the formation of off-flavor by preventing contamination. Therefore, SSS is a critical process to control the flavor of rice-flavor baijiu.

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Chapter 3. Correlation between key aroma and manufacturing processes of rice-flavor baijiu and awamori, Chinese and Japanese traditional distilled spirits

3-1. Introduction

Rice-flavor baijiu and awamori are traditional distilled spirits of China and Japan, respectively. Our previous study revealed several similarities in the manufacturing of both the distilled spirits (Yin et al., 2020a). There are two differences in the manufacturing process of the distilled spirits: the type of mold starter culture and the growth process. These differences must contribute to the characteristic flavors the distilled spirits. Rice-flavor baijiu possesses a sweet aroma and a clean mouthfeel (Zheng & Han, 2016), whereas awamori has a fruity, sweet, and characteristic mushroom-like flavor (Osafune et al., 2020). In our previous study, we investigated the similarities and differences in the flavor profiles of commercially manufactured baijiu and awamori. Rice-flavor baijiu contains a significant amount of short-chain acid ethyl esters such as ethyl isobutyrate and ethyl lactate and medium-chain fatty acid ethyl ester such as ethyl hexanoate (Yin et al., 2020b). In addition, it was found that ethyl lactate is the key volatile compound that is unique to rice-flavor baijiu. These key volatile compounds are potentially related to the solid-state saccharification (SSS). However, the commercial products have several minor differences in terms of the fermentation temperature and period as well as the rice cultivar used. Therefore, the key volatile compounds found in previous studies are derived from various factors in the manufacturing processes.

In this study, we aimed to investigate the correlation between the manufacturing process and the characteristic flavors in rice-flavor baijiu and awamori. Therefore, we prepared rice-flavor baijiu and awamori in our laboratory with SSS and koji making, respectively, under controlled conditions, including fermentation temperature and period, yeast strain, and distillation system. We identified and compared the flavor profiles and the volatile and major chemical compounds produced during both the fermentation processes.

3-2. Materials and Methods

3-2-1. Materials, reagents, and strains

All chemicals were acquired from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Nacalai Tesque Inc. (Kyoto, Japan). The yeast strain Kagoshima-5 (*Saccharomyces cerevisiae* Kagoshima no.5) (H5) was supplied by the Kagoshima Prefectural Brewing Association (Kagoshima, Japan) (Futagami et al., 2017). Yeast seed-culture was prepared by culturing Kagoshima-5 yeast in the YPD medium at 30°C for 48 h. Tane-koji (*A. luchuensis* mut. *kawachii*) starter was purchased from Kawachi Genichiro Shoten company (Kagoshima, Japan). Xiaoqu (Angel company, Hubei, China) for manufacturing rice-flavor baijiu was purchased from a local market in China. Polished rice was purchased from Hombo Shoten Co., Ltd (Kagoshima, Japan).

3-2-2. Mash preparation of awamori

Koji was prepared according to the method reported in our previous study (Yoshizaki et al., 2010). Briefly, polished rice (1.5 kg) was washed and soaked in water for 1 h. Excess water was drained for 1 h. The soaked rice was then steamed by a household steamer for 1 h to achieve the final moisture content of 37–38% (w/w), followed by cooling to approximately 45°C. The steamed rice was inoculated with tane-koji (15 g). The inoculated rice was incubated at 38°C under 95% humidity for 27 h after inoculation in the incubator (KCL-2000A, Tokyo Rikakikai Co., Ltd., Tokyo, Japan).

Then, it was further incubated at 35°C under 90% humidity for 16 h. The inoculated rice was stirred to cool the mass homogeneously at time points of 19 h, 23 h, and 27 h. Next, approximately 1.9 kg of the prepared koji (corresponding to 1.5 kg of polished rice) was divided into three equal portions. Koji was transferred to a flask containing 900 mL of water and 15 mL of the yeast seed-culture. The fermentation was carried out for 7 days at 30°C (Fig. 3-1).

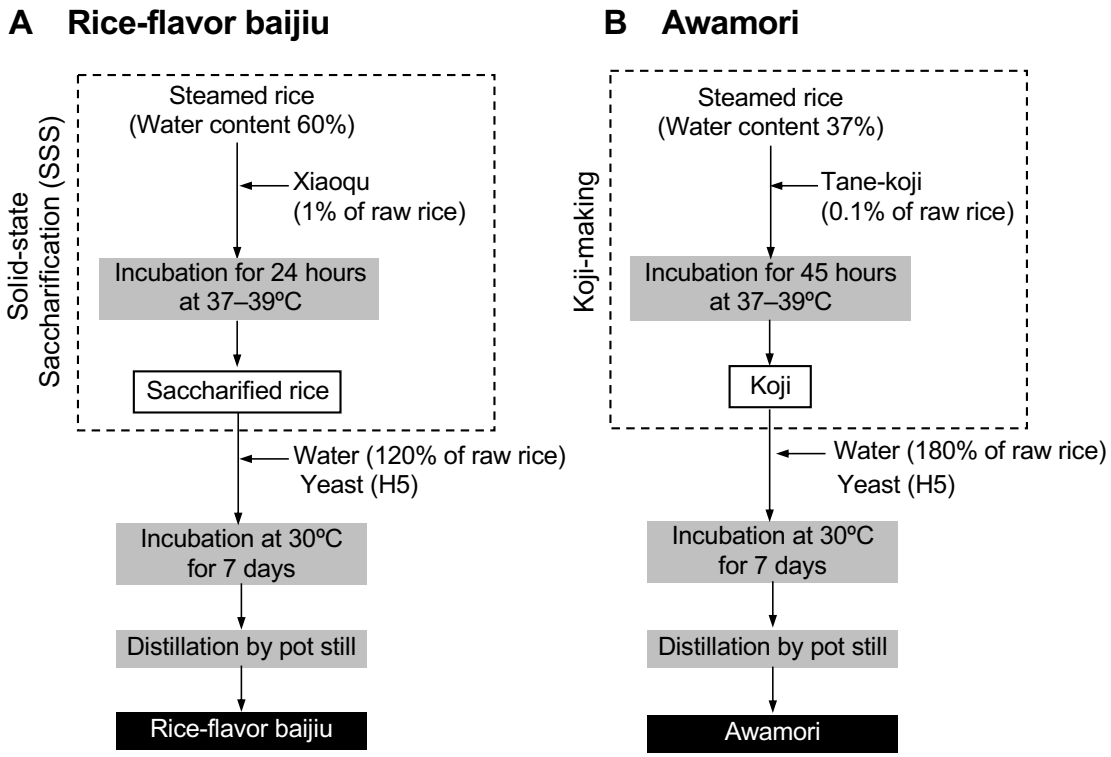

Fig. 3-1. Schematic illustration of the manufacturing processes of rice-flavor baijiu and awamori. (A) rice-flavor baijiu and (B) awamori.

3-2-3. Mash preparation of rice-flavor baijiu

The mash of rice-flavor baijiu was prepared according to the method reported in our previous study (Yin et al., 2020a). Briefly, polished rice (1.5 kg) was washed, soaked, and drained as mentioned in Section 2.2. The soaked rice was steamed by a household steamer twice to attain a final moisture content of 60% (w/w). The steamed rice was cooled to 40°C. The steamed rice (corresponding to 500 g of raw rice) was transferred to a separate flask, inoculated with 5 g xiaoqu, and then incubated for 24 h at 35°C. After incubation, 585 mL of water and 15 mL of yeast seed-culture were added to each flask, followed by fermentation for 7 days at 30°C (Fig. 3-1).

3-2-4. Monitoring of the alcohol fermentation

The alcohol fermentation was monitored by measuring the amount of CO_2 gas generated. The initial total weight of the containers was measured after preparing the mash. The total weight of the mash container was measured every 24 h during the fermentation process. The difference between the initial weight and the weight after incubation (the decrease in weight) was calculated to determine the amount of CO_2 gas generation. The integration curve for weight reduction was deduced based on these observations.

3-2-5. Distillation

Distilled spirit (awamori and rice-flavor baijiu) was prepared according to a

previous report (Shiraishi et al., 2016). Distilled spirit was obtained from single-batch distillation in a glass distillation apparatus (a glass pot still coupled to a glass column). Approximately 1 kg of fermented mash was distilled using the steam generated from water in a round-bottomed flask heated by a mantle heater. The distillate was then water-cooled. The endpoint of distillation was the point at which the alcohol content in the bundled distillate reached approximately 38%. The distillate was filtered and diluted to 25% by deionized water. The distilled spirit samples were then stored in a dark and cool place before the analysis.

3-2-6. Protease and acid carboxypeptidase assay

To 30 mL extraction buffer (100 mM acetate buffer [pH 5.0] containing 0.5% [w/v] NaCl), 20 g sample was added. The mixture was centrifuged at 13,700 *g* for 5 min (CF15RXII, Hitachi Ltd., Tokyo, Japan), and the supernatant was then collected. The pellet was resuspended with extraction buffer, and the supernatant was collected after another round of centrifugation. This cycle was repeated twice. Finally, the supernatants were pooled and adjusted to 100 mL with extraction buffer. Afterward, 10 mL extract was dialyzed with 10 mM acetate buffer (pH 5.0) at 4°C for overnight and adjusted to 20 mL with deionized water. The dialyzed solution was used as a crude enzyme to measure. The protease assay was adopted as described in our previous study (Yin et al., 2020a). One unit of protease activity was defined as the amount of enzyme required to liberate 1 mg of tyrosine from casein in 60 min at 35°C. Acid carboxypeptidase activity was measured using an acid carboxypeptidase assay kit (Kikkoman BioChemifa, Chiba, Japan) based

on the manufacturer's instructions. One unit of acid carboxypeptidase activity was defined as the amount of enzyme required to liberate 1 mol of L-alanine from carbobenzoxy-L-tyrosyl-L-alanine in 1 min at 37°C.

3-2-7. Short-chain acids analysis

The mash samples were prepared by centrifugation at 3,400 *g* for 10 min. The supernatant obtained was filtered through a 0.45 μm membrane filter. The concentrations of organic acids were determined by high-performance liquid chromatography (HPLC) (Prominence HPLC system, Shimadzu Co., Kyoto, Japan) using a conductivity detector (CDD-10A; Shimadzu Corp.). The analytic condition was adopted in our previous study (Rahayu et al., 2017). The stereospecificity of lactic acid was determined by the F-kit D/L-lactate assay (Roche Diagnostics K.K., Tokyo, Japan) based on the manufacturer's instructions.

3-2-8. Amino acids analysis

The mash samples were prepared as mentioned in the section 3-2-3. The concentrations of amino acids in the mash were determined by HPLC (Prominence HPLC system, Shimadzu Co.) using a fluorescence detector (RF-10AXL; Shimadzu Co.) by the post-column fluorescence derivatization method. The analytical conditions described in our previous study (Rahayu et al., 2017) were employed.

3-2-9. GC–MS with stir bar sorptive extraction

Mash was centrifuged at 4,800 *g* for 10 min. The supernatant was used for GC–MS analysis. The volatile compounds in the sample were collected and desorbed using the thermal desorption system (Gerstel K. K., Tokyo, Japan). The GC–MS system (GC; Agilent 6890N, MS; Agilent 5975B, Agilent Technologies Inc., CA, USA) was equipped with the Pure-WAX column (60 m × 0.25 mm I.D., 0.25 μm film thickness; GL Sciences Inc., Tokyo, Japan). GC-MS analysis with stir bar sorptive extraction was adopted in our previous study (Yin et al., 2020b). Identification of the volatile compounds was confirmed by comparison of the authentic standard, their mass spectra with those in the NIST05a mass-spectral database, and the retention index in the AromaOffice database (Nishikawa Keisoku Co., Ltd., Tokyo, Japan). Standard curves were constructed by linear regression of analyte peak areas versus known concentrations of each compound using authentic standards in our laboratory (Yin et al., 2020b).

3-2-10. GC–MS with a large volume static headspace sampling (LVSH)

The headspace volatile components were collected in LVSH system (Entech 7100A series; Entech Instruments Inc., Simi Valley, CA, USA). The distilled spirit samples (10 mL) were transferred to a 200 mL sample bottle. For quantitative determination, 1 mL of 1-pentanol (10 mg/L) was added to the samples as an internal standard. After incubation at 30°C, 100 mL of the headspace gas was vacuum-extracted from the sample bottle. GC-MS and LVSH-system conditions were adopted as described in our previous study

(Rahayu et al., 2017). The GC–MS system (GC; Agilent 7890A, MS; Agilent 5975C, Agilent Technologies Inc.) was equipped with the DB-WAX column (60 m × 0.25 mm I.D., 0.25 μm film thickness; Agilent Technologies Inc.). The identification of volatile compounds was constructed in the same manner with Section 2.9. The standard curve was constructed by linear regression of analyte peak areas versus known concentrations of 1-octen-3-ol in this study.

3-2-11. GC–MS for analysis of (+)-ethyl D-lactate and (−)-ethyl L-lactate

(+)-Ethyl D-lactate and (−)-ethyl L-lactate were identified and analyzed by GC–MS. The sample (2 mL) was mixed with 2 mL dichloromethane in a test tube. After mixing well, the dichloromethane layer was collected in another test tube and dehydrated by adding anhydrous sodium sulfate. The extract was transferred to a 1.5 mL vial and subjected to GC–MS. The GC–MS system (GC; Agilent 7890A, MS; Agilent 5975C, Agilent Technologies Inc.) was equipped with the CP-Chirasil Dec CB column (25 m × 0.25 mm I.D., 0.25 μm film thickness; GL Sciences Inc.). The identification of volatile compounds was constructed in the same manner with Section 2.9. Standard curves were constructed by linear regression of analyte peak areas versus known concentrations of (+)-ethyl D-lactate and (−)-ethyl L-lactate in this study.

3-2-12. Sensory evaluation

Rice-flavor baijiu and awamori were evaluated in terms of their aroma and taste.

Sensory profile analysis was conducted by 16 assessors (9 females and 7 males), from the Education and Research Center of Fermentation Studies of Kagoshima University. Most of the assessors were previously trained in sensory evaluation techniques by using the authentic compound and describing and recognizing the odor qualities of rice-flavor baijiu and awamori. The assessors discussed and reached a consensus regarding a list of relevant sensory attributes related to aroma and taste defined by the National Research Institute of Brewing in Japan. The intensity of each characteristic was evaluated on a scale of 0–5 (0 = not detected, 1 = very weak intensity, 2 = weak intensity, 3 =moderate intensity, 4 = strong intensity, and 5 = very strong intensity). The results of the sensory profile analysis were averaged for each aroma and plotted on a spider web diagram.

3-2-13. Measurement of odor threshold

The measurement of odor threshold was based on ASTM (E679-04) (2011) (ASTM International, 2011). This analysis was conducted by 12 assessors (7 females and 5 males) from Kagoshima University. A series of test samples were prepared in 25% (v/v) ethanol solution in six concentration steps, which increased by a factor of two per step. Triangular tests were conducted using the same solvent as the control. The best estimate threshold of each assessor were calculated as the geometric mean of the last missed concentration and the next higher concentration. The group threshold was calculated as the geometric mean of the best estimate threshold.

3-2-14. Fatty acid oxygenase activity

The fatty acid oxygenase activity in saccharified rice and koji was assayed using the procedure described by Narisawa et al. with minor modifications (2019). Saccharified rice and koji (500 mg) were resuspended in 200 μL of 50 mM phosphate-K buffer (pH 6.0) containing 1 mM dithiothreitol and broken with 0.3 g of glass beads (size 0.35–0.50 mm). Then, 1300 μl of 50 mM phosphate-K buffer (pH 6.0) containing 1 mM dithiothreitol was added and mixed well. The solution was transferred to a new plastic tube and centrifuged at 13,700× *g* for 5 min at 4°C. The supernatant was used for the assay. Fatty acid oxygenase activity was determined using linoleic acid as the substrate. A mixture of 250 μL of a substrate solution (8.09 mM linoleic acid in 50 mM borate buffer, pH 9.0), 2.9 mL of 50 mM phosphate-K buffer (pH 6.0), and 100 μL sample were added in a cuvette. The absorbance at 234 nm at 25°C was recorded for 3 min. One unit of fatty acid oxygenase activity was defined as the quantity of enzyme required to increase the absorbance by one unit per min per gram of fresh sample at 234 nm at 25°C.

3-2-15. Statistical analysis

Statistical analyses were performed using IBM® SPSS® Statistics version 27 software (IBM, NY, USA). Analysis of variance was performed to compare samples. Significant differences ($P < 0.05$) between means were determined using Student's *t*-tests.

3-3. Results and Discussion

3-3-1. Manufacturing of rice-flavor baijiu and awamori and the observation of fermentation

Rice-flavor baijiu and awamori were produced by the typical conditions to the industrial manufacturing process (Fig. 3-1). The mashes of rice-flavor baijiu and awamori were fermented for 7 days. During the first 5 days, the rate of fermentation, in the case of awamori mash was greater than that of rice-flavor baijiu (Fig. 3-2). However, from the fifth day onward, the difference in the rate of fermentation of the two mashes reduced and was comparable by the end of the fermentation. The total amounts of alcohol produced, total sugar content (residual sugars), and yeast cell numbers in the fermented mash were substantively similar (Table 3-1). These results indicated that both mashes were finally fermented to the same level despite the different fermentation speed.

The major acid in awamori mash was citric acid, where that in rice-flavor baijiu mash was lactic acid (Table 3-1). The acetic acid concentration in awamori mash was 4-times greater than that of rice-flavor baijiu mash. The mash pH of rice-flavor baijiu was higher than that of awamori. In wine brewing, the acetic acid production level by yeast was influenced by various factors, such as pH, the sugar content of the mash, or the yeast strain (Caridi et al., 1999; Radler, 1993; Shimazu & Watanabe, 1981). Therefore, the higher acetic acid production may be induced by the low pH in the awamori mash.

The amino acid content in the mash affects the fermentation rate and yeast growth. Therefore, we analyzed the amino acid composition. The total amino acid contents of awamori mash were 10-times greater than that of rice-flavor baijiu (Fig. 3-3A). The

protease and acid carboxypeptidase activities, mainly coming from the mold, were also determined. The protease activity in the saccharified rice was approximately 6 times lower than that in koji (Fig. 3-3B). The acid carboxypeptidase activity in the saccharified rice was not detected and that in koji was 4.37 ± 0.31 U/g dry weight, respectively (Fig. 3-3B). Therefore, the higher amino acid content in awamori mash must be the result of a higher amount of proteolytic enzymes produced by *A. luchuensis* when compared to that by *R. oryzae*. Moreover, the higher rate of fermentation during the early stage of fermentation, in the case of awamori, may be due to the higher content of amino acids.

Table 3-1. Analysis of the fermented mash.

	Rice-flavor baijiu	Awamori
Mash alcohol (%)	15.2 ± 0.3	17.1 ± 0.1
Total alcohol (ml)	227.0 ± 3.8	226.6 ± 1.7
Total sugar contents (%)	1.72 ± 0.00	1.37 ± 0.03
Viable cell numbers (cells/ml)	6.7×10^{7} ± 4.6×10^{6}	2.3×10^{8} ± 6.6×10^{7}
Total cell numbers (cells/ml)	2.5×10^{8} ± 2.7×10^{7}	3.4×10^{8} ± 1.0×10^{8}
Mash pH	3.71 ± 0.01	3.53 ± 0.01
Acetic acid (mM)	2.10 ± 0.31	8.80 ± 0.28
Citric acid (mM)	0.49 ± 0.02	57.97 ± 0.21
Lactic acid (mM)	50.47 ± 0.73	4.80 ± 0.17

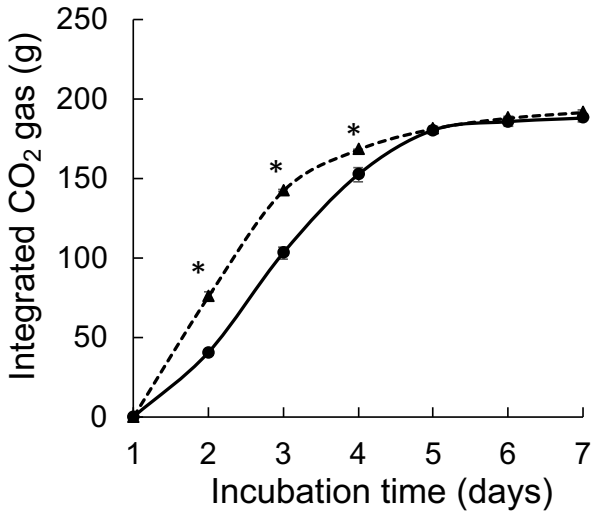

Fig. 3-2. Alcohol fermentation of the mashes of rice-flavor baijiu and awamori. The alcohol fermentation was monitored by observing the amount of CO_2 gas generated. The total weight of the mash container was measured each day. The difference between the initial weight and the weight after incubation (the decrease in weight) was used to determine the amount of CO_2 gas generation. The integration curve for weight reduction was plotted on a graph. Rice-flavor baijiu and awamori are indicated by a solid line and a dotted line, respectively. Significant difference (*$P < 0.01$) between means of each days were determined using Student's *t*-test.

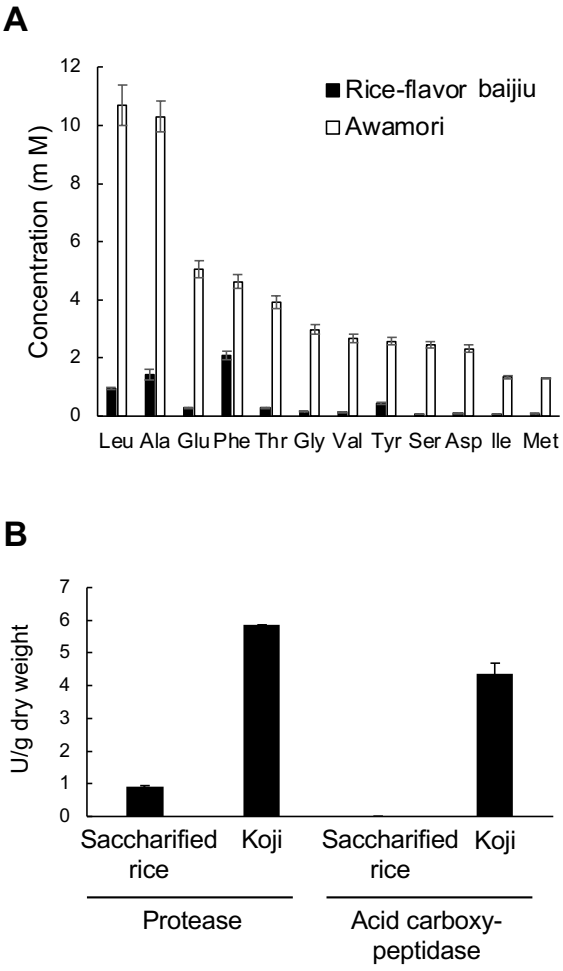

Fig. 3-3. Amino acid contents in the fermented mash. Solid bars and open bars indicate rice-flavor baijiu and awamori, respectively.

3-3-2. The flavor properties of (+)-ethyl D-lactate and (−)-ethyl L-lactate and the contents in the distilled spirits

In the case of flavors, sensory properties of the volatile compounds containing asymmetric carbon atoms are known well to differ between isomers. A well-known example is the aroma difference of the enantiomers of carvone. The (*R*)-(−)- and (*S*)-(+)-enantiomers have the aromas of caraway and spearmint, respectively (Russell & Hills, 1971). Furthermore, it has been reported that the aroma threshold of *R*-ethyl 2-hydroxy-4-methyl pentanoate (126 μg/L) was almost twice that of the *S* form (55 μg/L) (Lytra et al., 2012). There are two chiral forms of ethyl lactate; (+)-ethyl D-lactate and (−)-ethyl L-lactate. However, the flavor properties and aroma thresholds of (+)-ethyl D-lactate and (−)-ethyl L-lactate have not been measured separately to date. Therefore, in this study, we determined their aroma threshold and quality. The aroma threshold (−)-ethyl L-lactate was 1.2-times lower than that of (+)-ethyl D-lactate (Table 3-2). Although both the compounds have a common sweet, fruity, and green leaves-like aromas, the nuance was slightly different. (+)-Ethyl D-lactate has a peach- and citrus-like aromas, whereas (−)-ethyl L-lactate has an apple-like or milky aromas.

3-3-3. Sensory properties and volatile compounds of the distilled spirits

We evaluated the sensory properties of the two distilled spirits by sensory evaluation. It was confirmed that rice-flavor baijiu had significantly stronger alcohol, fruity, and floral aromas and tended to have stronger ester and sweet aromas. On the other

hand, awamori tended to have a strong koji-like, sour, oily, and cereal-like aromas and a strong astringent mouthfeel (Fig. 3-4). It was confirmed that both distilled spirits showed different odor and taste profiles.

The volatile compounds in the two distilled spirits were analyzed by GC–MS. In this study, 7 alcohols, 20 esters, 5 acids, 3 aldehydes, and 3 others were detected. (+)-Ethyl D-lactate and (−)-ethyl L-lactate were present in higher amounts in rice-flavor baijiu than in awamori. In rice-flavor baijiu, the concentration of (−)-ethyl L-lactate was 13-times greater than that of (+)-ethyl D-lactate (Table 3-2). The only OAV of (−)-ethyl L-lactate was >1. There were 16 compounds with OAV >1 including (−)-ethyl L-lactate (Table 3-2). Six of those were at almost the same level between both the distilled spirits (namely, ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, octanoic acid, and 2-nonenal). It was considered that the concentrations of these compounds were not directly correlated with the manufacturing process of both the distilled spirits. Six of the 16 compounds were higher in rice-flavor baijiu than in awamori (namely, isobutyl alcohol, isoamyl alcohol, 1-octanol, *β*-phenylethyl alcohol, (−)-ethyl L-lactate, and isoamyl acetate). Isobutyl alcohol and isoamyl alcohol have an alcohol-like aroma; *β*-phenylethyl alcohol has a rose-like aroma; and (−)-ethyl L-lactate and isoamyl acetate have a fruity aroma (Fig. 3-4). These aroma characterizations were consistent with the results of the sensory evaluation of rice-flavor baijiu. Four of the 16 compounds were higher in awamori than in rice-flavor baijiu (namely, 1-octen-3-ol, ethyl 2-methyl-butyrate, acetic acid, and dimethyl trisulfide (DMTS)). The acetic acid content in awamori was much higher than that in rice-flavor baijiu, which explains the perception of a sour aroma in awamori in the sensory evaluation. In addition, the tendency of the acetic acid content in

the distilled spirits was consistent with the acetic acid concentrations in the mash (Table 3-1).

1-octen-3-ol in awamori is known to derived from koji (Fukuda & Han, 2016). Koji prepared by *A. luchuensis* has a mushroom-like aroma caused by 1-octen-3-ol. Therefore, the aroma of 1-octen-3-ol is often expressed as "koji-like" for awamori. The stronger koji-like aroma in awamori indicates higher 1-octen-3-ol content. It is reported that fatty acid oxygenase PpoC in *A. luchuensis* and PpoA and PpoC in *A. nidulans* are involved in the biosynthesis of 1-octen-3-ol (Brodhun et al., 2010; Kataoka et al., 2020). Therefore, we measured the fatty acid oxygenase activity in saccharified rice and koji. The fatty acid oxygenase activity in koji was approximately 50 times higher than that in saccharified rice (Fig. 3-5A). These results are consistent with the tendency of 1-octen-3-ol content in rice-flavor baijiu and awamori. The fatty acid oxygenase in *R. oryzae* is not reported as far as we found. Protein BLAST using the amino acid sequences of *A. luchuensis* PpoA and PpoC identified three homologous proteins (accession numbers KAG1140824.1, KAG1146499.1, and KAG1146413.1) in *R. oryzae*. However, the amino acid sequences of *R. oryzae* Ppo homologous proteins (679, 695 and 688 amino acid residues, respectively) is shorter than that of *A. luchuensis* and *A. luchuensis* mut. *kawachii* fatty acid oxygenases (Fig. 3-5B). N-terminal region including the heme peroxidase domain is well conserved in the *A. luchuensis* PpoA and PpoC and *R. oryzae* Ppo homologous proteins. However, *R. oryzae* homologous proteins do not have the cytochrome P450 domain unlike the Ppo proteins of *A. luchuensis* and *A. luchuensis* mut. *kawachii*. These facts imply that *R. oryzae* Ppo homologous proteins might have a different function to *Aspergillus* Ppo proteins.

The tendency of rice-flavor baijiu to contain higher contents of isobutyl alcohol, isoamyl alcohol, *β*-phenylethyl alcohol, and ethyl lactate than awamori was consistent with that in a commercial product (Yin et al., 2020b). Therefore, these compounds were associated with the SSS process. Higher alcohol, such as isobutyl alcohol, isoamyl alcohol, and *β*-phenylethyl alcohol, is produced by yeast via the Ehrlich pathway or the amino acid biosynthesis pathway (Äyräpää, 1965). When the content of amino acid in the mash is low, the amino acid biosynthetic pathway is upregulated. It has been reported that the higher alcohol production by yeast is achieved via amino acid biosynthesis in the case of Japanese-distilled spirit brewing (Shiraishi et al., 2016; Shiraishi et al., 2017). The content of amino acids in the mash of rice-flavor baijiu was less than that of awamori (Fig. 3-3). Therefore, it was suggested the amino acid biosynthesis pathway leads to the production of higher amounts of alcohols. These results showed that SSS contributes to the production of a high concentration of alcohol because the proteolytic enzymes activities were low in saccharified rice and that the low amino acid content in the mash promoted higher alcohol production by stimulating amino acid biosynthesis pathways in yeast. In addition, koji produces a high concentration of 1-octen-3-ol because koji has higher fatty acid oxygenase activity, which is essential for the production of 1-octen-3-ol, than saccharified rice.

The ethyl hexanoate, isoamyl acetate, and acetic acid contents in this study were higher in awamori than in rice-flavor baijiu. However, in the commercial products, the amounts of these compounds are higher in rice-flavor baijiu than in awamori (Yin et al., 2020b). Especially, we reported that the higher acetic acid concentration is one of the characteristic compounds in rice-flavor baijiu as distinct from awamori of commercial

products. Therefore, the tendency varied. Ethyl hexanoate, isoamyl acetate, and acetic acid production level are influenced by the yeast strain (Saerens et al., 2008; Piendl & Geiger, 1980). In many alcoholic beverage, high content of acetic acid is undesirable because it causes a significant reduction in quality (Cordente et al., 2013; Zhang et al., 2012). On the other hand, high ethyl hexanoate content is an important property in Chinese distilled spirits (Shen, 2003). Therefore, low acetic acid content in the commercial awamori and high ethyl hexanoate content in the commercial rice-flavor baijiu may depend on the yeast strain selected for manufacturing.

DMTS was higher in awamori than in rice-flavor baijiu. DMTS in rice wine is also formed during storage and fermentation from the precursor related with methionine salvage pathway of yeast (Wakabayashi et al., 2013). In addition, it is reported that the DMTS content in rice wine is increased by the high temperature during fermentation (Sasaki et al., 2014). Therefore, the variations in yeast strain and fermentation conditions directly affect the ethyl hexanoate, isoamyl acetate, acetic acid, and DMTS content.

Table 3-2. Volatile compounds in rice-flavor baijiu and awamori.

Compounds	Odor description	RI	m/z	Concentration (μg/L)					Odor threshold (μg/L)	OAV		Ref
				Rice-flavor baijiu		Awamori				Rice-flavor baijiu	Awamori	
				Mean	SD	Mean	SD					
(Alcohol)												
Isobutyl alcohol	Penetrating, wine-like	1086	43	334,241	60,079	67,386	8,774	**	40,000	8.4	1.7	1
1-Butanol	Similar to amyl alcohol	1128	56	4,990	663	3,563	458	*	5,000	1>	1>	2
Isoamyl alcohol	Alcohol-like, pungent	1188	55	287,383	25,747	106,440	6,120	**	30,000	9.6	3.5	1
1-Hexanol	Herbal, woody	1323	56	172	20	12	1	**	5,200	1>	1>	2
1-Octanol	Fresh, orange-rose odor	1524	56	1,116	62	58	2	**	1,100	1.0	1>	2
1-Octen-3-ol§	Mushroom-like	–	57	26.6	4.3	83.5	15.7	*	20	1.3	4.2	3
β-Phenylethyl alcohol	Rose	1858	91	42,879	524	20,521	709	**	10,000	4.3	2.1	1
(Ester)												
(+)-Ethyl D-lactate¶	Sweet, fruity	–	45	1,336	277	4,993	1,243	**	15,400	1>	1>	This study
(−)-Ethyl L-lactate¶	Sweet, fruity	–	45	17,131	1199	893	202	**	12,500	1.4	1>	This study
Ethyl butyrate	Fruity, pineapple	1009	71	507	24	506	34		20	25	25	1
Ethyl 2-methyl-butyrate	Green-fruity, apple	1026	102	13	2	51	4	**	1	13	51	1
Ethyl hexanoate	Fruity, banana, pineapple	1211	88	282	26	356	15	*	5	56	71	1
Ethyl heptanoate	Fruity, brandy-like	1307	88	2.2	0.2	2.9	0.1	**	220	1>	1>	4
Ethyl octanoate	Fruity, floral	1423	88	2,429	160	2,752	95	*	2	1,215	1,376	1
Ethyl nonanoate	Fruity, fatty, oily	1508	88	22	2	39	1	**	1,300	1>	1>	3
Ethyl decanoate	Fruity, oily, brandy-like	1624	88	2,112	141	1,404	68	**	200	10.6	7.0	5
Ethyl dodecanoate	Floral, fruity	1815	88	184	24	88	17	**	500	1>	1>	4
Ethyl palmitate	Waxy sweet	2220	88	275	43	336	67		>14,000	1>	1>	2
Ethyl Oleate	Floral	2440	55	70	6	51	8	*	870	1>	1>	2
Isoamyl acetate	Fruity, banana	1104	43	1,780	133	622	83	**	30	59	21	1
Isoamyl hexanoate	Fruity, banana, apple	1436	70	0.31	0.03	0.39	0.01	**	1,400	1>	1>	2
Isoamyl octanoate	Fruity	1635	70	10.1	0.7	3.8	0.2	**	125	1>	1>	5
Isoamyl decanoate	Waxy, fruity	1831	70	1.6	0.1	0.6	0.1	**	>5,000	1>	1>	2
Isobutyl acetate	Fruity, floral	983	56	46	2	13	2	**	3,400	1>	1>	2
Isobutyl hexanoate	Fruity, cocoa-like	1327	99	7.2	0.8	1.3	0.4	**	–	–	–	–
Hexyl acetate	Apple, cherry, floral	1248	56	8.1	1.6	1.2	0.1	**	670	1>	1>	3
Octyl acetate	Fruity	1449	43	102	13	4.7	0.1	**	50,000	1>	1>	6
(Acid)												
Acetic acid†	Sour	–	–	25,446	3381	104,753	4,120	**	28,000	1>	3.7	7
Octanoic Acid	Unpleasant, faint	2005	60	1,242	73	685	46	**	500	2.5	1.4	5
n-Decanoic acid	Fatty, unpleasant	2215	73	1,491	30	922	56	**	15,000	1>	1>	1
Dodecanoic acid	Fatty	2425	73	408	18	151	5	**	15,000	1>	1>	2
Tetradecanoic acid	Wax, oily	2633	73	1,279	85	412	67	**	>12,000	1>	1>	2
(Aldhyde)												
2-Octenal	Fatty, green	1396	55	2.5	0.4	1.7	0.6		3	1>	1>	8
2-Nonenal	Fatty, violet-like	1501	70	16	2	20	4		0.08	197	248	8
Phenylacetaldehyde diethyl acetal	Rose	1682	103	13	1	80	13	**	–	–	–	–
(Other)												
2-Pentylfuran	Green bean, metallic,	1204	81	3.1	0.5	4.3	0.8		6	1>	1>	9
2-Undecanone	Peach	1567	58	6.2	0.5	3.1	0.0	**	7.0	1>	1>	8
Dimethyl trisulfide	Fresh onion	1342	126	nd	–	0.62	0.21		0.2	–	3	1

nd, not detected. Significant differences (*$P < 0.05$; **$P < 0.01$) between means were determined using Student's *t*-test. §Compound analyzed by LVSH. ¶Compound analyzed by Chiral-column. †Compound analyzed by HPLC. Odor threshold obtained from references: [1] Guth, 1997, [2] Salo et al., [3] Welke et al., 2014, [4] Zea et al., 2001, [5] Ferreira et al. 2000, [6] Li et al., 2008, [7]Lee et al., 2000, [8]Buttery et al., 1988, [9]Buttery et al., 1990.

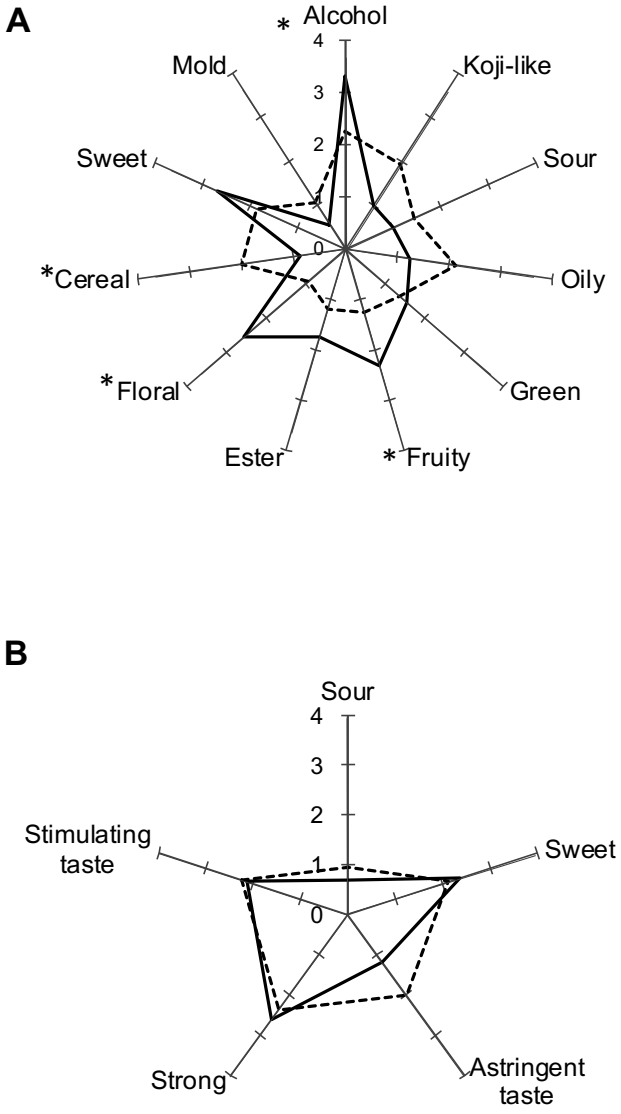

Fig. 3-4. Sensory evaluation. (A) Aromas and (B) tastes and mouthfeel of rice-flavor baijiu and awamori. The solid line and dotted line indicate rice-flavor baijiu and awamori, respectively. Significant difference (*P < 0.05) between means were determined using Student's t-test.

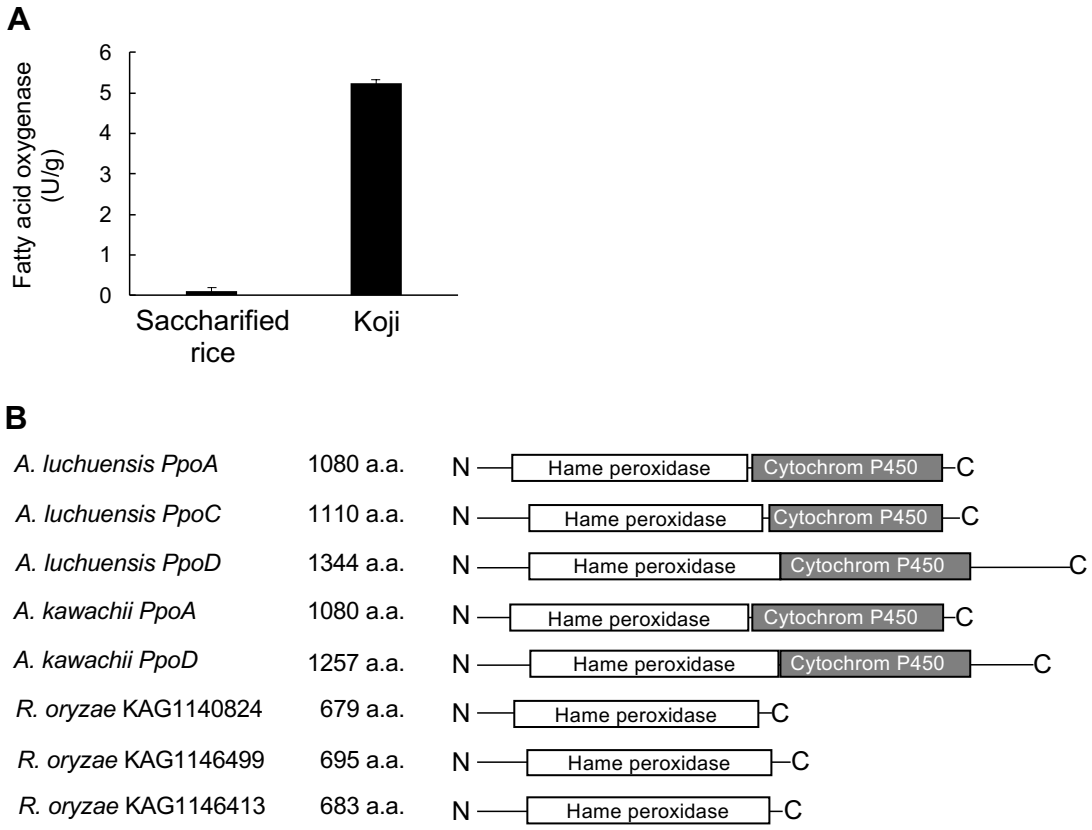

Fig. 3-5. Fatty acid oxygenase analysis. (A) Fatty acid oxygenase activity in saccharified rice and koji. (B) Domain analysis of *Aspergillus luchuensis* Ppo and *Rhizopus oryzae* Ppo homologous proteins using InterPro (https://www.ebi.ac.uk/interpro/). Amino acid (a.a.) sequence data was submitted to the NCBI database under the following accession numbers: *A. luchuensis* PpoA, GAT25971; *A. luchuensis* PpoC, GAT23542; *A. luchuensis* PpoD, GAT24722; *A. luchuensis* mut. *kawachii* (*A. kawachii*) PpoA, XP_041544978; *A. luchuensis* PpoD, XP_041549055; *R. oryzae* Ppo homologus protein, KAG1140824, KAG1146499, KAG1146413.

3-3-4. L(+)-lactic acid and D(−)-lactic acid contents in the mash

Our results showed that the ethyl lactate content was strongly related to the lactic acid content in the fermented mash. The (−)-ethyl L-lactate and (+)-ethyl D-lactate contents may be affected by the L(+)-lactic acid and D(−)-lactic acid contents in the mash. Therefore, we prepared the mash of rice-flavor baijiu and awamori again and measured the L(+)-lactic acid and D(−)-lactic acid contents in the mash. In the mash of rice-flavor baijiu, the L(+)-lactic acid contents at day 2 and at the end of fermentation were 13–22-times higher than the D(−)-lactic acid contents (Table 3-3). This indicates that the main lactic acid in the mash of rice-flavor baijiu was L form and its concentration gradually decreased during the fermentation. In the mash of awamori, the D(−)-lactic acid content was higher than the L(+)-lactic acid contents. Therefore, it was confirmed that the tendency of rice-flavor baijiu mash to contain higher contents of L(+)-lactic acid and awamori mash to contain higher contents of D(−)-lactic acid were consistent with that of (−)-ethyl L-lactate acid and (+)-ethyl D-lactate in the distilled spirits. Therefore, we concluded that L(+)-lactic acid was mainly produced during SSS and used for the production of ethyl lactate during fermentation. *R. oryzae* has been reported to directly produce almost optically pure L(+)-lactic acid from starch (Hang, 1989). D(+)-Lactic acid is generally produced with lactic acid bacteria such as *Lactobacillus* species (Yáñez et al., 2003). Therefore, the L(+)-lactic acid in the mash of rice-flavor baijiu must have been produced by *R. oryzae.* These results showed that SSS contributes to the production of a high concentration of ethyl lactate by supplying a large amount of lactic acid in the mash of rice-flavor baijiu.

This study is the first to focus on the relationship between the key aromatic compounds and the manufacturing processes of rice-flavor baijiu and awamori. The results could facilitate the understanding of the individual flavor characteristics of rice-flavor baijiu and awamori and the steps involved in the development of these characteristics.

Table 3-3. DL(±)-lactic acid contents in the mash.

	Rice-flavor baijiu		Awamori	
	Day 2	Final day	Day 2	Final day
D(−)Lactic acid (mM)	1.7 ± 2.9	2.2 ± 0.8	1.6 ± 0.1	2.6 ± 0.2
L(+)Lactic acid (mM)	37.1 ± 3.6	27.1 ± 2.1	0.8 ± 0.2	1.2 ± 0.5

3-4. Summary

We investigated the correlation between the flavor profiles of rice-flavor baijiu and awamori and their manufacturing process. Sensory evaluation revealed that rice-flavor baijiu had stronger alcoholic, sweet, floral, and fruity aromas; while awamori had a strong sour, koji-like, oily, and cereal-like aromas. We measured the concentrations, aroma threshold, and aroma quality of two isomers of ethyl lactate. The aroma thresholds of (+)-ethyl D-lactate and (−)-ethyl L-lactate were determined to be 15,400 μg/L and 12,500 μg/L, respectively. Both the compounds have common sweet, fruity, and green leaves-like aromas and slightly different nuance; (+)-ethyl D-lactate has a peach- and citrus-like aromas, whereas (−)-ethyl L-lactate has an apple-like and milky aromas. In rice-flavor baijiu, (−)-ethyl L-lactate is the main form of ethyl lactate, possibly due to the higher concentration of L(+)-lactic acid in the mash. Among the compounds which were >1 of OAV, higher alcohols, (−)-ethyl L-lactate and isoamyl acetate contents were higher in rice-flavor baijiu than in awamori, and 1-octen-3-ol and acetic acid were higher in awamori than in rice-flavor baijiu. These aroma characterizations were consistent with the sensory evaluation of both the distilled spirits. Isobutyl alcohol, isoamyl alcohol, *β*-phenylethyl alcohol, ethyl lactate, 1-octen-3-ol were related to the mold type used in manufacturing. On the other hand, it was shown that the production of isoamyl acetate, acetic acid, and DMTS in both distilled spirits correlated with the yeast strain, fermentation conditions, and storage condition.

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Chapter 4. Discussion

Rice-flavor baijiu is a traditional Chinese liquor. In this study, the flavor profile and volatiles presented with or without the SSS were investigated to reveal the effects of SSS process on the quality of rice-flavor baijiu in chapter 2. In addition, we compared the flavor profiles Chinese rice-flavor baijiu and Japanese awamori—traditional liquors with similar manufacturing processes in chapter 3.

These results were clearly shown that ethyl lactate was one of the characteristic odor compounds in rice-flavor baijiu and SSS process of rice-flavor baijiu making contributes to produce the high concentration of ethyl lactate. In our previous study, we showed that lactic acid bacteria were hardly detected in saccharified rice throughout SSS, and *Rhizopus* sp. grew vigorously during SSS (Yin et al., 2020a). In addition, we confirmed that *Rhizopus* sp. that grew in saccharified rice had lactic acid production ability. It was strongly indicated that a large amount of lactic acid in the mash with SSS reduced the pH of the mash and repressed the growth of lactic acid bacteria. Therefore, during the fermentation, the amount of lactic acid is mainly produced by *Rhizopus* sp. In addition, during fermentation, an amount of lactic acid increased in both mash with SSS and without SSS. It was shown that a part of lactic acid in the mash might be also produced by yeast. On the other hands, during the fermentation of mash without SSS, the initial pH was close to neutral. Thus, it was suggested that lactic acid bacteria can grow

in the mash without SSS. In this study, we investigated that (−)-ethyl L-lactate was the main form of ethyl lactate in rice-flavor baijiu. We used the commercial xiaoqu which is prepared by cultivating *Rhizopus oryzae* of pure culture in the cereal powder. Therefore, it is expected that the lactic acid bacteria and other bacteria is very few in the xiaoqu (Yin et al., 2020a). However, xiaoqu for using in the industry may have more complex bacterial flora because the jiuqu, most used starter for Chinese liquor, has very complex bacterial flora. Daqu is revealed that the complex bacterial flora is affected to the production of unique volatile compounds (Fan et al., 2018).

In our previous study, we found that the acetic acid content of commercial awamori is lower than that of commercial rice-flavor baijiu. (Yin et al., 2020b). Therefore, we consider that high acetic acid was one of characterization for distinguishing from awamori. We found that the concentration of acetic acid was higher in rice-flavor baijiu with SSS. This result shows that SSS process contribute to produce high concentration of acetic acid in rice-flavor baijiu. On the other hand, the amount of acetic acid in awamori made in our laboratory was more than that in the rice-flavor baijiu. It was not accorded with the result of commercial samples. These results suggests that SSS was not be related directly to the high concentration of acetic acid in commercial rice-flavor baijiu but contributed to increase the acetic acid.

β-phenylethyl alcohol was also considered to be the characteristic aroma component of rice-flavor baijiu. In studies of distilled spirits prepared from rice (sake and

shochu), many reports showed that lower amino acid contents in the fermented mash promote the production of higher alcohol containing *β*-phenylethyl alcohol. While, higher amino acid contents in the fermented mash repress higher alcohol production (Setoguchi et al., 2019; Shiraishi et al., 2021; Takamine et al.1989). In the chapter 3, we confirmed that the amino acid content in awamori was higher than that in rice-flavor baijiu, and the content of *β*-phenylethyl alcohol might be less than that in rice-flavor baijiu. This result was consistent with the results of the literature. On the other hand, in the chapter 2, we found that the content of *β*-phenylethyl alcohol in the rice-flavor baijiu with SSS was higher, and the concentration of amino acids in the rice-flavor baijiu with SSS was also higher than that of the rice-flavor baijiu without SSS. Therefore, it can be concluded that the production of *β*-phenylethyl alcohol was related to the SSS process. In the future, we will explore the relationship between *β*-phenylethyl alcohol content and amino acid concentrations in rice-flavor baijiu.

In the present study, we demonstrate the role of the SSS process in the production of characteristic flavor compounds in rice-flavor baijiu. These results could facilitate the efficient manufacture of rice-flavor baijiu.

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