Identification of characteristic aroma components and study on quality stabilization in rice-flavor *baijiu*

小曲米酒の特徴香気成分の同定と品質安定化に関する研究

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印　璇　　令和 2 年

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Chapter 1. Introduction

1-1. *Baijiu*

Baijiu is a Chinese traditional liquor with a high ethanol content ranging from 38 to 65 vol%. It has a production history of at least 2000 years (Liu and Sun, 2018). *Baijiu* is a major part of the Chinese food industry, since the high consumption with a market value of approximately $88.5 billion in 2017 (Qian *et al*., 2019). In general, the *baijiu* is produced from the grains such as sorghum and *jiuqu*. *Jiuqu* is a starter for fermentation. It is prepared by culturing microorganisms on grains and contained the microbial enzymes for brewing. Therefore, *jiuqu* plays a similar role to *koji* on *shochu* (a traditional liquor in Japan) and malt on whiskey (Zhu and Tramper, 2013). In addition, *jiuqu* contains the yeast. *Jiuqu* can be categorized in *daqu*, *xiaoqu*, and *fuqu* based on its preparation method (Gou *et al*., 2015).

Baijiu has various production methods. It imparts the diversity to the flavors. This concept of flavor types of *baijiu* was first created to facilitate the evaluation of liquor samples in 1979 (Han *et al*., 2015). At that time, the notions of strong-, sauce-, light-, and rice-flavor *baijiu* were created as the four basic flavor types. Nowadays, there are 12 flavor types (Zhang *et al*., 2018).

1-2. Flavor characteristics and manufacturing processes of the basic flavor types

1-2-1. Strong-flavor *baijiu*

Strong-flavor *baijiu* is also called *Luzhou*-flavor *baijiu*. "*Luzhou*" derives from "*Luzhou Laojiao*" which was the first brand to be rated as high-quality strong-flavor *baijiu* in the national liquor appraisal. The strong-flavor *baijiu* has the characteristics of fragrant aroma, soft mouthfeel, and endless aftertaste (Zheng and Han, 2016). The characteristic aroma components in this type are identified from various samples (Table 1-1). The representative aroma compounds are predominantly ethyl hexanoate in harmonious balance with ethyl lactate, ethyl acetate, and ethyl butanoate (Liu and Sun, 2018).

Strong-flavor *baijiu* is made from grains (sorghum, rice, glutinous rice, wheat, and corn) and medium-temperature *daqu* prepared at 50~60ºC during the process of *daqu* preparation (Zheng *et al*., 2011). The fermentation and distillation methods are characterized by carrying out in the solid-state. The detailed production method refers to the diagram of the manufacturing process (Fig. 1-1). The steamed grains are mixed with *daqu* powder and fermented for 45~90 days in a mud pit under the solid-state (Jin *et al*., 2017). The fermented mash is also distilled in solid-state by steaming. During

distillation, the raw materials for next fermentation are mixed with the fermented mash. Therefore, the distillation of fermented mash and the steaming of raw materials occur at the same time. This distillation method makes the liquor grain aroma (Xiao *et al*., 2014). After the distillation, the residue is mixed with *daqu* powder again and returns to the mud pit as a next mash. In this way, the fermentation and distillation are continuously and circularly carried out (Liu and Sun, 2018). The mud pit is one of the most prominent features of strong-flavor *baijiu* making. The mud pit cellar size is about 12 m^3 (Fig. 1-1). In this mud, a lot of microbes naturally survive. These microbes are well researched because it is interested in complexity. It is revealed that some microbe contributes to form various aroma components, so far (Xu *et al*., 2017). For example, the genera of *Caproiciproducens*, *Lactobacillus*, *Clostridium* IV, unclassified *Clostridiaceae* I, and unclassified *Anaerobrancaceae* produced organic acids such as propionic acid, lactic acid, and caproic acid. These organic acids were the precursors of important aroma components (Tao *et al*., 2014; Liu *et al*., 2017). Especially, *Clostridium* sp. refers to caproic acid-producing bacteria in the research of strong-flavor *baijiu*. It contributes to a large amount of ethyl hexanoate (ethyl caproate) which is most important volatile compound in strong-flavor *baijiu* (Tao *et al*., 2014).

1-2-2. Sauce-flavor *baijiu*

Sauce-flavor *baijiu* is also called *Moutai*-flavor *baijiu*. "*Moutai*" derives from famous product name of sauce-flavor *baijiu* in the world and it is rated as high-quality sauce-flavor *baijiu* in the National Liquor Appraisal. Sauce-flavor *baijiu* has full-body, mellow, and sweet taste with a lingering aftertaste and a characteristics sauce-like flavor (Zhu *et al*., 2007). The characteristic aroma components are well researched (Table 1-2). It was associated with a high content of pyrazines in sauce-flavor *baijiu* compared to other types (Xiao *et al*., 2014; Fan *et al*., 2007).

Sauce-flavor *baijiu* is made from sorghum and high-temperature *daqu* prepared at 60~70ºC during the process of *daqu* preparation (Zheng *et al*., 2011). The fermentation and distillation are carried out in the solid-state like strong-flavor *baijiu*. The steamed sorghum is mixed with the *daqu* powder, and the mixture is stacked on the ground for approximately 5 days. Then, the mixture is transferred into the stone cellar and fermented for the 1 month (Fig. 1-2A). After the fermentation, the mash is distilled with an equal amount of sorghum in solid-state by steaming (Fig. 1-2B). The first distillate is returned to the next fermentation mash, and not become a liquor. The residue after distillation is mixed with the *daqu* powder for re-fermentation and re-distillation for 7 cycles (Fig. 1-2C). Thus, there have eight distillations in the sauce-flavor *baijiu*

production, the distillates are collected from the second distillation. These distillates are batched together and become a liquor. The liquor is stored for at least five years before the packaging process (Wang *et al*., 2019). It was summarized that various microorganisms in the high-temperature *daqu* and the stacking fermentation process made a great contribution to the flavor of the liquor (Wang *et al*., 2019). Among these microorganisms, *Bacillaceae* was thought to be associated with the sauce-like flavor and dominated in the stacking fermentation (Wang *et al*., 2015b). It is shown that *Bacillus subtilis* and *B. licheniformis* isolated from high-temperature *daqu* can produce pyrazines such as 2,3-dimethylpyrazine, 2,3,5-trimethylpyrazine, and 2,3,5,6-tetramethylpyrazine (Zhu *et al*., 2010; Zhang *et al*., 2013). Meanwhile, pyrazines can be also produced by the Maillard reaction between saccharide and amino residues from the repeated operations of production processes (Fan and Xu, 2012). In addition, the liquor with the longer-term storage exhibited the sauce-like flavor more prominently (Fan *et al*., 2011).

1-2-3. Light-flavor *baijiu*

Light-flavor *baijiu* is also called Fen-flavor *baijiu*. “Fen” derives from famous product name of light-flavor *baijiu.* It is also rated as high-quality light-flavor *baijiu* in

the National Liquor Appraisal. Light-flavor *baijiu* is divided into three types according to the type of *jiuqu* for manufacturing; *daqu* light-flavor *baijiu*, *fuqu* light-flavor *baijiu*, and *xiaoqu* light-flavor *baijiu*. Light-flavor *baijiu* has a pure and mild flavor, mellow, sweet, and refreshing aftertaste (Zheng and Han, 2016). The characteristic aroma compounds in light-flavor *baijiu* compared with strong- and sauce-flavor *baijiu* are revealed as ethyl acetate, 2-methylpropyl acetate, isoamyl acetate, isoamyl lactate, ethyl decanoate, diethyl butanedioate, 2-phenethyl acetate, 3,4-dihydro-2H-1-benzopyran-2-one 1-dodecanol, ethyl decanoate, 3-methyl-1-butanol, and thymol (Xiao *et al*., 2016).

The fermentation and distillation are carried out in the solid-state. The *daqu* light-flavor *baijiu* is made from sorghum and low-temperature *daqu* as the starter prepared at 40~50ºC during the process of *daqu* preparation (Zheng *et al*., 2011). The fermentation is carried out in the earthen jars of about 0.5 m^3 volume for 1 month (Fig. 1-3). After fermentation, the mash is distilled in solid-state by steaming. Then, the residue after distillation is mixed with the *daqu* powder to be fermented and distilled again. These processes are repeated one more time. The distillates for two times are batched together and become a liquor. The *fuqu* light-flavor *baijiu* is fermented in a cellar for 7~21 days. The fermented mash is also distilled in solid-state. This fermentation and distillation are not repeated and carried out a single batch. The single distillate becomes a liquor. The *xiaoqu* light-flavor *baijiu* is fermented 7 days in a

cement pit (Zhang *et al*., 2017). This fermentation and distillation are carried out the same way of *fuqu* light-flavor *baijiu*. In these three types of light-flavor *baijiu*, it is considered that *baijiu* produced using *xiaoqu* or *fuqu* contains less flavor than that produced using *daqu* (Table 1-3) (Zheng and Han, 2016). This could be ascribed to the significantly different microbial communities in the three types of *jiuqu* and the low fungal diversity in *xiaoqu* and *fuqu* (Gou *et al*., 2015). Likewise, the low-temperature *daqu* can contribute to the aroma of the light-flavor *baijiu*, which is mainly ethyl acetate, in balance with considerable levels of ethyl lactate (Zheng *et al*., 2011). There are reports that the microbial community structure (Zheng *et al*., 2015), the representative metabolites or biomarkers characteristic (Wu *et al*., 2009), and the contribution of predominant hydrolyase (Liu *et al*., 2018) were distinct among high-temperature, medium-temperature, low-temperature *daqu*. In the analyses of extracts from three types of *daqu* using ^{1}H nuclear magnetic resonance (NMR) spectroscopy, acetate was the biomarkers of low-temperature *daqu* (Wu *et al*., 2009). This suggested that microorganisms producing acetate played an important role in the low-temperature *daqu*. Meanwhile, the lactic acid bacteria (LAB) of *Weisella confusa* is found at high abundance in low-temperature *daqu* (Zheng *et al*., 2015). These corresponded well with the fact that the represented aroma of light-flavor *baijiu*.

1-2-4. Rice-flavor *baijiu*

Rice-flavor *baijiu* is also called "*Mijiu*". "*Mi*" and "*jiu*" mean "rice" and "liquor" in Chinese. Rice-flavor *baijiu* has a characteristic sweet-honey aroma. It is reported that ethyl acetate, 3-methylbutyl acetate, ethyl octanoate, acetic acid, propanoic acid, butanoic acid, 2-phenylethanol, 4-ethylguaiacol, and γ-nonalactone were the important aroma compounds in rice-flavor *baijiu* compared with other type of *baijiu* (Fan *et al*., 2019).

Rice-flavor *baijiu* is made from rice and *xiaoqu.* The steamed rice is mixed with *xiaoqu* powder (left in Fig. 1-4A). The mixture is incubated for 20~30 hours in the solid-state. This process is called as solid-state saccharification. Then, water is added, then the mash is fermented in a ceramic pottery jars or the closed stainless-steel vessel under the liquid-state for approximately 1 week (Fig. 1-4B) (Wang, 2003a). Finally, the fermented mash is distilled in the liquid-state like Japanese *shochu* (Shen, 1998). This fermentation and distillation are carried out a single batch. The single distillate becomes a liquor. Like these, rice-flavor *baijiu* greatly differs from three types described above in some respect; a kind of raw material, the type of *jiuqu,* and fermentation and distillation state. This characteristic process could contribute to the characteristic flavor of rice-flavor *baijiu*. However, it has been not revealed yet.

1-3. The research purposes

Rice-flavor *baijiu* has a unique manufacturing method and flavor characteristics described above. We are interested in the characterization of flavor in rice-flavor *baijiu* compared to world liquor, and also in the relationship between the flavor and manufacturing process. However, there is little attention and research, because rice-flavor *baijiu* has a low share of *baijiu* market in China. There are few relevant scientific theories as the guidance basis, resulting in low production efficiency and unstable quality (Hu *et al*., 2019).

Shochu, Japanese traditional distilled liquor, has been produced from about 17th century. In the early production, the fermented mash was sometimes corrupted (Sameshima, 2004). Until now, high-quality *shochu* can be stably produced. It has been through the efforts of generations, and its production technology is gradually mature under the guidance of scientific theory (Takamine, 2015). Thus, the production technology of rice-flavor *baijiu* must be also improved and developed.

In this study, we aimed to understand the flavor characteristics to be observed and the production method scientifically. First, we have analyzed the aroma compounds in commercial products as we understand the characteristic flavor of rice-flavor *baijiu* compared to *awamori* and *kome-shochu*. *Awamori* and *kome-shochu* are made from rice

by liquid-state fermentation and distillation like rice-flavor *baijiu*. Next, we have investigated the role of the solid-state saccharification process in making of rice-flavor *baijiu* which is found only in rice-flavor *baijiu* and unique procedure.

TABLE 1-1. The characteristic aroma compounds of the strong-flavor *baijiu*.

Aroma compound	Reference	Aroma compound	Ref
Ester			
ethyl hexanoate	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	butyl hexanoate	2, 10
ethyl butyrate	1, 2, 3, 4, 5, 6, 8, 9, 10	ethyl 3-phenylpropanoate	4, 8
ethyl valerate	1, 2, 4, 5, 8, 9, 10	ethyl nonanoate	1
ethyl octanoate	1, 2, 4, 5, 8, 9, 10	3-methylbutyl hexanoate	5
ethyl acetate	3, 6, 8	ethyl isobutyrate	8
ethyl lactate	3, 6, 7	isoamyl acetate	8
ethyl decanoate	1, 9	ethyl isovalerate	9
ethyl heptanoate	1, 7	ethyl phenylacetate	9
ethyl 3-methylbutanoate	2, 10	hexyl hexanoate	9
Acid			
hexanoic acid	1, 2, 4, 5, 6, 7, 8, 9, 10	octanoic acid	1
butyric acid	1, 4, 5, 6	nonanoic acid	1
valeric acid	1, 5, 7	decanoic acid	1
acetic acid	1, 6	undecanoic acid	1
propionic acid	1	3-methylbutanoic	5
heptanoic acid	1		
Alcohol			
1-butanol	1, 3, 7, 8	1-octanol	1
1-hexanol	1, 5, 7, 8	1-nonanol	1
isopentanol	3, 5, 7	1-propanol	3
1-pentanol	1	isobutanol	3
1-heptanol	1		
Others			
1, 1-diethoxy-3-methylbutane	2, 5, 10	4-methylphenol	4
ac*et al*	3, 6	γ-nonalactone	4
ac*et al*dehyde	3, 6	4-ethylphenol	5
3-methyl butanal	8, 9	4-ethylguaiacol	5
methylal	1	4-methylguaiacol	5
furfural	1	1, 1-diethoxy-2-methylpropane	5
dimethyl disulfide	1	1, 1-diethoxy ethane	5
s-methyl thiobutyrate	1	nonanal	8

1. Yao *et al*., 2015; 2. Fan and Qian, 2006a; 3. Fan and Xu, 2000; 4. Zhao *et al*., 2018a; 5. Fan and Qian, 2006a; 6. Tang *et al*., 2006; 7. Feng *et al*., 2012; 8. Wang *et al*., 2015a; 9. Xiao *et al*., 2014; 10. Fan and Qian, 2006b.

TABLE 1-2. The characteristic aroma compounds of the sauce-flavor *baijiu*.

Aroma compound	Reference	Aroma compound	Ref	Aroma compound	Ref
Ester					
ethyl acetate	1, 2, 3, 4, 5, 6	ethyl nonanoate	1, 2	ethyl 2-hydroxy-3-methylbutanoate	1
ethyl butyrate	1, 2, 3, 4, 5, 6	3-methylbutyl hexanoate	1, 2	ethyl 2-methylpentanoate	4
ethyl pentanoate	1, 2, 3, 4, 5, 6	ethyl hexadecanoate	1, 6	ethyl 2-hydroxyhexanoate	4
ethyl hexanoate	1, 2, 3, 4, 5, 6	ethyl myristate	1, 6	ethyl 4-methylpentanoate	4
ethyl lactate	1, 2, 3, 5, 6	diethyl butanedioate	2, 6	ethyl 4-oxopentanoate	4
ethyl octanoate	1, 2, 3, 5, 6	2-methylpropyl acetate	2, 3	ethyl E,Z-2,4-decadienoate	4
ethyl 2-methylpropanoate	1, 2, 3, 4, 5	hexyl acetate	2, 5	2-phenylethyl butanoate	4
ethyl propanoate	1, 2, 3, 5	hexyl hexanoate	2, 5	2-phenylethyl hexanoate	5
ethyl heptanoate	1, 2, 4, 5	ethyl formate	1	isopentyl butanoate	5
ethyl decanoate	1, 2, 5, 6	ethyl hexadecanoate	1	butyl butyrate	6
ethyl 3-methylbutanoate	1, 2, 3, 4	methyl hexanoate	1	butyl lactate	6
3-methylbutyl acetate	1, 2, 3, 6	pentyl hexanoate	1	isoamyl formate	6
butyl hexanoate	1, 2, 4, 5	ethyl undecanoate	2		
ethyl 2-methylbutyrate	2, 3, 4, 6	ethyl dodecanoate	2		
2-phenylethyl acetate	2, 3, 4, 6	ethyl 2-furoate	2		
ethyl phenylacetate	2, 4, 5, 6	3-methylbutyl butanoate	2		
ethyl benzoate	1, 2, 4	heptyl acetate	2		
ethyl 3-phenylpropanoate	2, 3, 4	propyl hexanoate	2		
Acid					
hexanoic acid	1, 2, 3, 4, 5	heptanoic acid	2, 4, 6	propanoic acid	5, 6
butyric acid	1, 2, 4, 5, 6	octanoic acid	1, 2	decanoic acid	4
pentanoic acid	1, 2, 5, 6	2-methylpropanoic acid	1, 5	2-phenylacetic acid	4
3-methylbutanoic acid	1, 3, 4, 5	4-methylpentanoic acid	1, 5		
acetic acid	1, 4, 5, 6	nonanoic acid	2, 4		
Alcohol					
1-octanol	1, 2, 3, 5, 6	1-pentanol	1, 5	1-decanol	2
3-methylbutanol	1, 2, 4, 5, 6	1-octen-3-ol	2, 3	2-methyl-1-butanol	3
2-methyl-1-propanol	3, 4, 5, 6	4-ethylphenol	2, 4	2-phenyl-1-propanol	4
1-hexanol	2, 3, 5	3-octanol	2, 5	2,6-dimethoxyphenol	4
4-methylphenol	2, 4, 5	1-propanol	3, 5	1-phenyl-1-ethanol	4
2-phenylethanol	2, 4, 6	1-butanol	5, 6	4-methyl-2-methoxyphenol	4
1-heptanol	1, 2	2-heptanol	1	phenol	4
2-octanol	1, 2	2-hexanol	1	furfuryl alcohol	6
2-nonanol	1, 2	2-pentanol	1	benzyl alcohol	6
2-butanol	1, 5	1-nonanol	2		

TABLE 1-2. Continued.

Aroma compound	Reference	Aroma compound	Ref	Aroma compound	Ref
Pyrazine					
2,3,5-trimethylpyrazine	2, 3, 4, 6, 9	2,3-dimethyl-5-ethylpyrazine	1, 4	2-methyl-6-vinylpyrazine	4
2,3,5,6-tetramethylpyrazine	1, 2, 4, 9	2,3,5-trimethyl-6-ethylpyrazine	1, 4	2-methoxy-3-butylpyrazine	4
2,5-dimethylpyrazine	1, 2, 9	1,1-diethoxy-2-methylpropane	1, 4	2,3-dimethyl-Z-5-propenylpyrazine	4
2,6-dimethylpyrazine	1, 4, 5	1,1-diethoxy-3-methylbutane	1	3-(1-methylethyl)-2-methoxypyrazine	4
2-ethyl-6-methylpyrazine	1, 4	1,2-dimethoxy-3-methylbenzene	4	3,5-dimethyl-2-pentylpyrazine	4
Others					
1,1-diethoxyethane	1, 2, 4, 5, 6	2-acetylfuran	2	4-hydroxy-3-methoxybenzaldehyde	4
dimethyl trisulfide	1, 2, 3, 5, 7	2-acetyl-5-furoate	2	1-(5-methyl-2-furanyl)-1-propanone	4
furfural	1, 2, 5, 6	α-terpineol	2	dihydro-5-propyl-2(3H)-furanone	4
β-damascenone	2, 3, 8	octanal	2	dihydro-5-hexyl-2(3H)-furanone	4
benzaldehyde	2, 5, 6	nonanal	2	dihydro-5-octyl-2(3H)-furanone	4
benzothiazole	1, 4	2-heptanone	2	dihydro-5-(Z-2-octenyl)-2(3H)-furanone	4
2-pentanone	1, 4	2-octanone	2	3-hydroxy-4,5-dimethyl-2(5H)-furanone	4
phenylac*et al*dehyde	1, 5	2-nonanone	2	2-methylpropanal	5
dimethyl disulfide	1, 7	2-decanone	2	3-hydroxy-2-butanone	6
5-methyl-2-furfural	2, 4	2-undecanone	2	2-furfurylthiol	7
geranyl acetone	2, 4	2-pentadecanone	2	methional	7
3-methylbutanal	2, 5	γ-nonalactone	2	S-methyl thioacetate	7
β-ionone	3, 8	dimethyl sulfide	4	methanethiol	7
linalool	3, 8	2-furancarboxaldehyde	4	ethanethiol	7
3-octen-2-one	1	ac*et al*dehyde	5	α-ionone	8
acetophenone	2	1-phenyl-1-hexanone	4	citral	8
naphthalene	2	Z-whiskylactone	4		

1. Zhu *et al*., 2007; 2. Fan *et al*., 2011; 3. An *et al*., 2019; 4. Fan *et al*., 2012; 5. Wang *et al*., 2014; 6. Niu *et al*., 2017a; 7. Chen *et al*., 2017; 8. Wang *et al*., 2016; 9. Fan *et al*., 2007.

TABLE 1-3. The characteristic aroma compounds in the different types of light-flavor *baijiu*.

		light-flavor *baijiu* type		
	Characteristic aroma components	*daqu*[I]	*fuqu*[II]	*xiaoqu*[III]
Esters	ethyl acetate	✓	✓	✓
	ethyl butanoate	✓		
	ethyl pentanoate	✓	✓	
	ethyl hexanoate	✓	✓	✓
	ethyl lactate	✓	✓	
	ethyl octanoate	✓	✓	✓
	ethyl decanoate	✓	✓	✓
	ethyl phenylacetate		✓	
	ethyl 2-methylpropanoate	✓	✓	
	ethyl 3-methylbutanoate	✓		
	ethyl 3-phenylpropanoate	✓		
	2-methylpropyl acetate	✓		
	2-phenylethyl acetate		✓	
	3-methylbutyl acetate	✓	✓	
Acids	acetic acid	✓		
	butanoic acid	✓	✓	
	pentanoic acid	✓	✓	
	hexanoic acid	✓	✓	
	2-methylpropanoic acid	✓	✓	
	3-methylbutanoic acid	✓	✓	
Alcohols	1-propanol			✓
	1-butanol	✓		
	1-hexanol	✓		
	2-methylpropanol	✓	✓	✓
	3-methylbutanol	✓	✓	✓
	1-octen-3-ol	✓	✓	
Aldehyde	hexanal, nonanal, and decanal	✓		
	3-hydroxy-2-butanone		✓	
	phenylac*et al*dehyde	✓	✓	
Others	1,1-diethoxyethane	✓		
	γ-nonalactone	✓		
	β-damascenone	✓	✓	
	geosmin	✓		
	guaiacol, 4-ethylguaiacol, and dimethyl trisulfide		✓	

I.Gao *et al*., 2014; II.Niu *et al*., 2017b; III.Ma *et al*., 2016. The check marks represent the presence of a characteristic aroma compound.

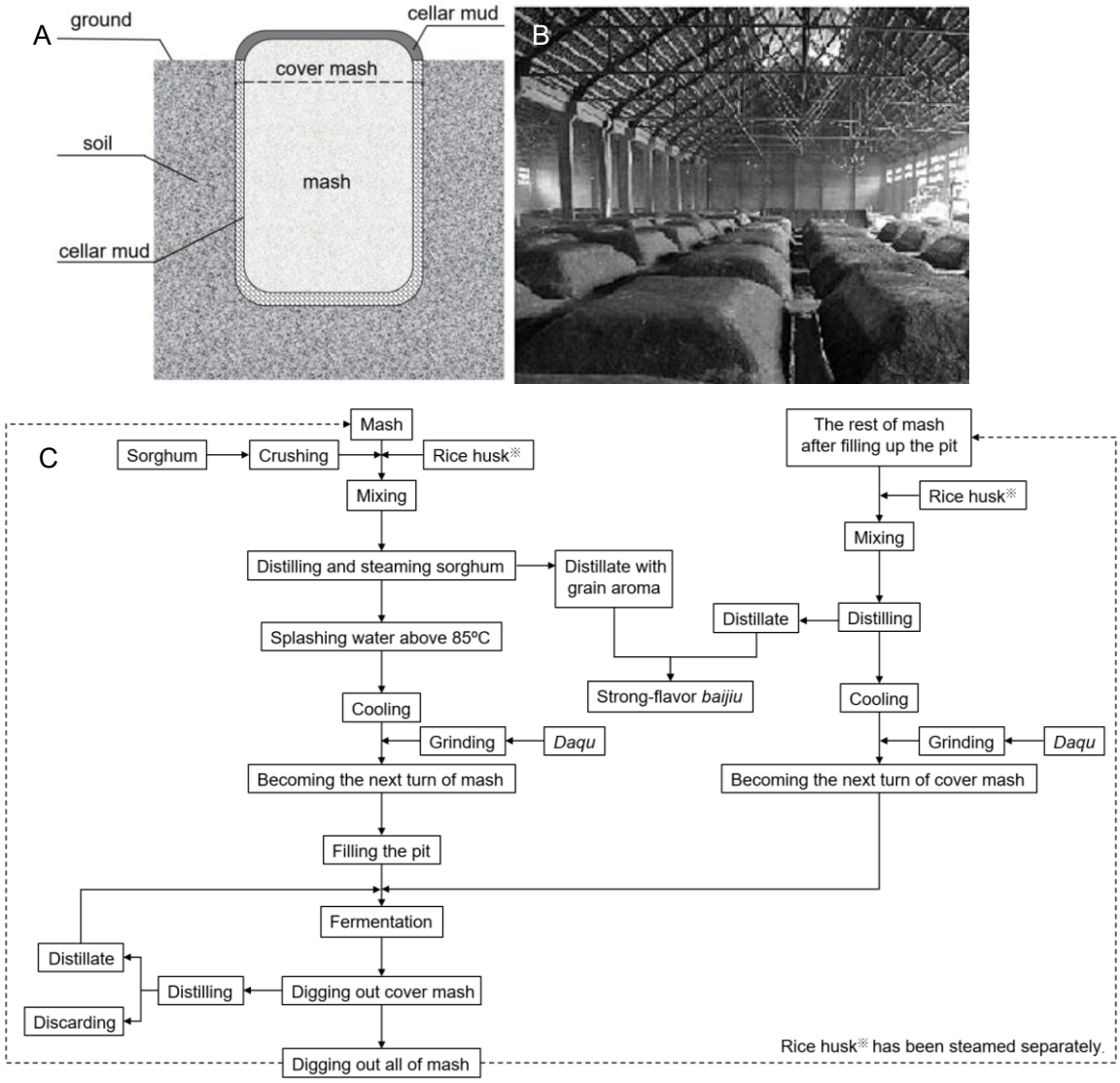

FIG. 1-1. The mud cellar and the production method in strong-flavor *baijiu*. A, the sectional drawing of mud cellar adapted from Li *et al*. (2013). B, photo of the cellar filled up the mash taken from Li *et al*. (2013). C, the diagram of the manufacturing process.

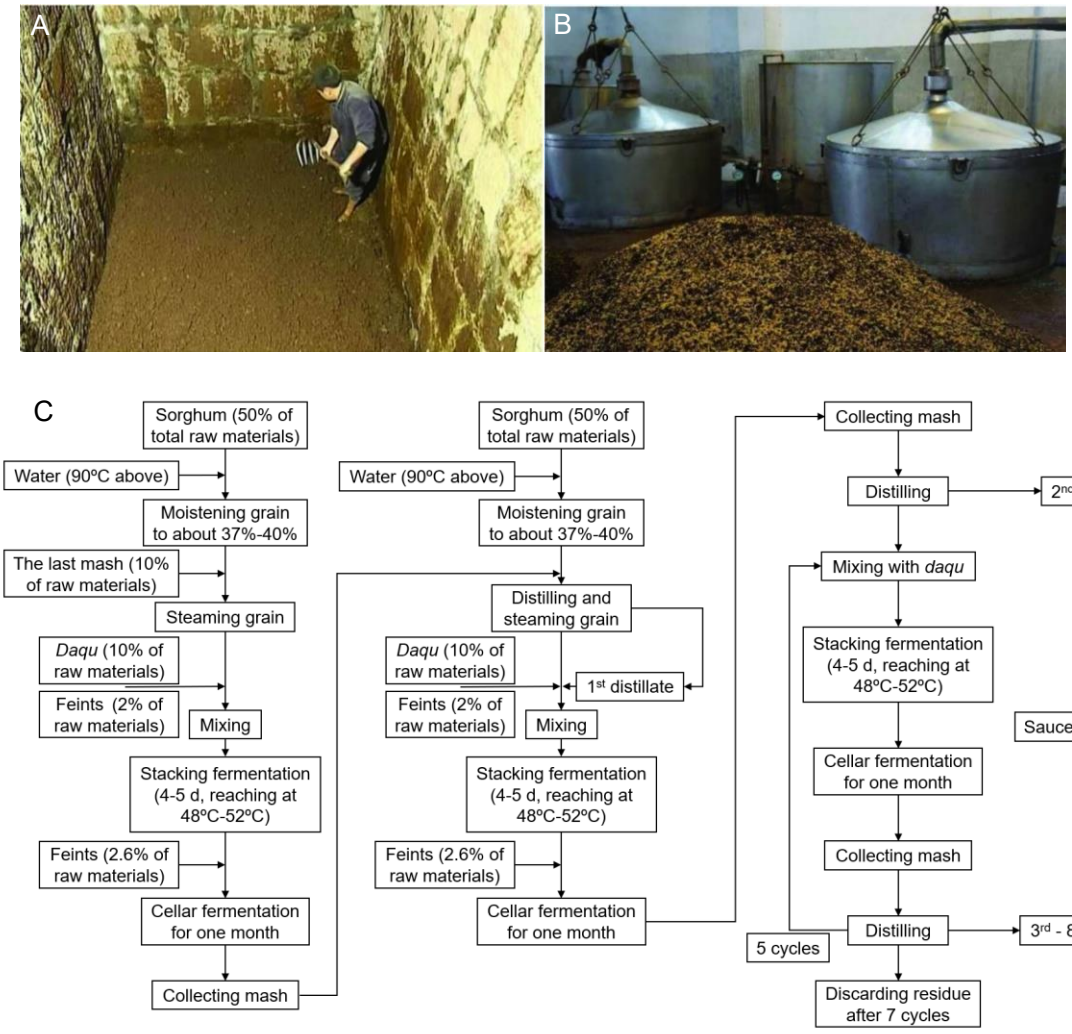

FIG. 1-2. The stone cellar, the distillation vessel, and the production method in sauce-flavor *baijiu*. A, photo of the cellar being filled with the mash taken from Fan *et al*. (2012). B, photo of the *baijiu* distiller taken from Fan *et al*. (2012). C, the diagram of the manufacturing process adapted from Wang *et al*. (2019).

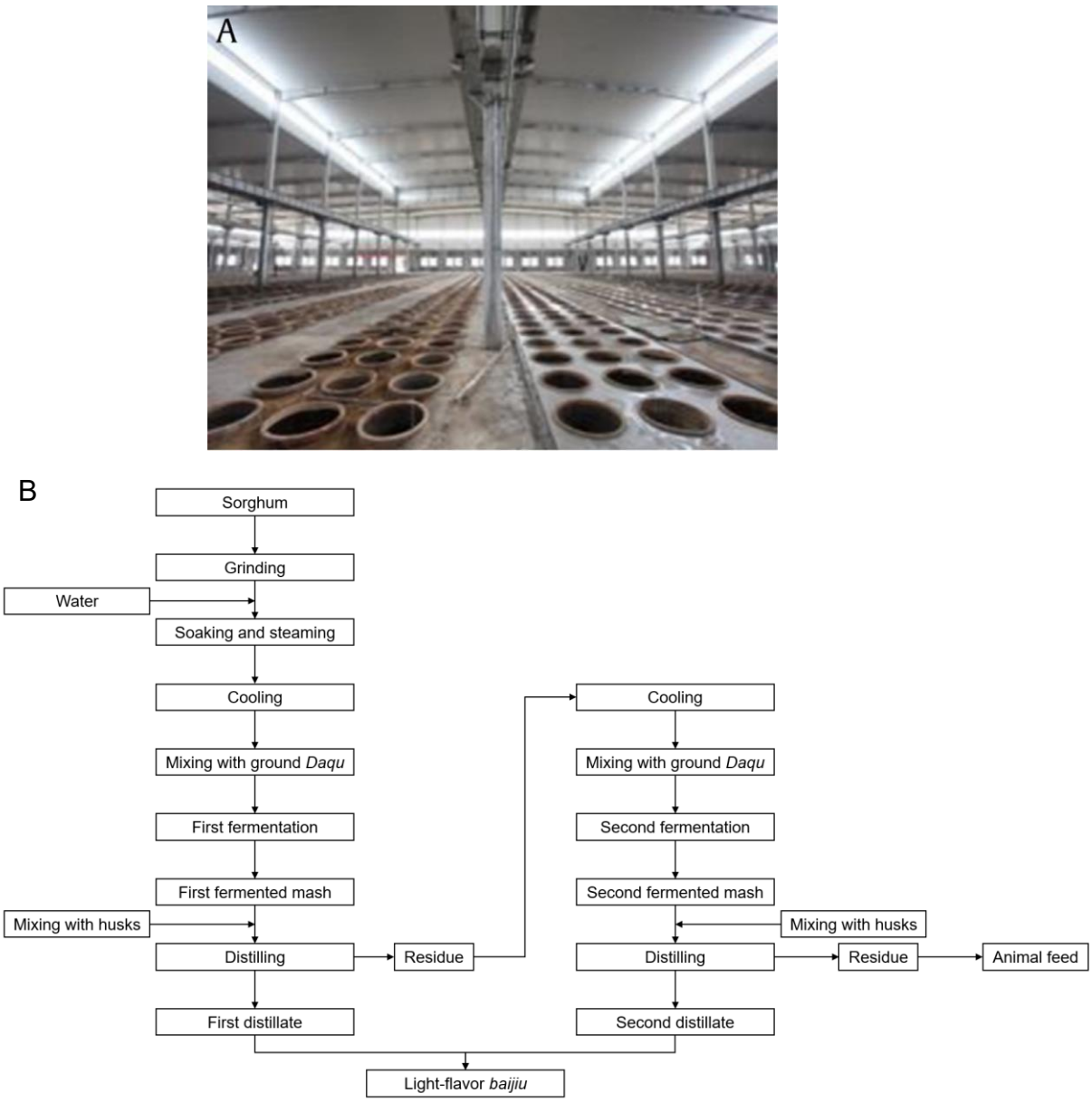

FIG. 1-3. The fermentation vessel and the production method in light-flavor *baijiu*. A, photo of the fermentation earthen jars taken from Zheng and Han (2016). B the diagram of the manufacturing process adapted from Zheng and Han (2016).

FIG. 1-4. The starter *xiaoqu* and the traditional and modern fermentation vessel in rice-flavor *baijiu*. A, photos of the different kinds of *xiaoqu* taken from Fan *et al*. (2019). B, photos of the ceramic pottery jars for the traditional fermentation and the closed stainless-steel tank for the modern fermentation taken from Cui and Li (2011).

Chapter 2. The characterization of flavor compounds of rice-flavor *baijiu*

2-1. Introduction

Baijiu is a traditional Chinese distilled liquor and rice-flavor *baijiu* is mainly produced in southern China. Rice-flavor *baijiu* is made from rice as the sole ingredient and *xiaoqu* as a fermentation starter. *Xiaoqu* is made from rice powder and rice bran (Zheng and Han, 2016). A few types of microorganism are present in *xiaoqu,* including *Rhizopus* sp., *Mucor* sp., and yeasts (Zheng and Han, 2016).

The manufacturing process of rice-flavor *baijiu* involves semi-solid-state fermentation (semi-SSF) (Zheng and Han, 2016). SSF is performed by a solid matrix in the absence of free water. Briefly, after steaming rice, a small amount of *xiaoqu* is added to it and mixed well (Fig. 2-1). This solid mixture is incubated for 20–30 h at room temperature. After incubation, water is added, and the mixture is fermented in liquid state for about 10 days. Finally, the fermented mash is distilled in liquid state.

This manufacturing process of rice-flavor *baijiu* is very different from that of the other type of Chinese *baijiu* (Fig. 2-1). Other types of Chinese *baijiu*, such as strong-flavor *baijiu*, sauce-flavor *baijiu* and light-flavor *baijiu*, are typically made of several cereals, such as sorghum, wheat and rice, and complex microorganisms from the natural

mixed starter *daqu*. SSF is performed for several months in a pit or earthen jars (Zheng and Han, 2016). Many microorganisms, such as caproic acid–producing bacteria, live in mud pits and earthen jars (Xiong *et al*., 2010), and this complicated microorganism flora contributes to the liquor's flavors (Wang *et al*., 2014a).

On the other hand, the manufacturing process of rice-flavor *baijiu* is similar to that of *awamori* and *kome-shochu*, two traditional Japanese distilled liquors. *Awamori* uses *tane-koji* as a fermentation starter, which is prepared from the *koji* mold (*Aspergillus luchuensis*). Rice *koji* is a solid culture of this *koji* mold grown on steamed rice for approximately 42 h. Rice *koji,* water and yeast seed culture are mixed and fermented in liquid state for about 10 days. After fermentation, the mash is distilled in liquid state. *Kome-shochu* is made from rice and rice *koji* prepared from *A. luchuensis* mut. *kawachii* (Fig. 2-1). It is produced using the same method as *awamori*, except that the main ingredients are added separately after the first fermentation.

Awamori has light, sweet and mushroom-like flavors, whereas *kome-shochu* has a characteristic fruity flavor (Miyamoto, 2018; Hayashida, 1998). Isobutyl alcohol, 1-octen-3-ol, nerolidol and S-methyl thioacetate are the key volatile compounds in *awamori* compared to other Japanese *shochu* types (Fukuda *et al*., 2016). In particular, 1-octen-3-ol is what imparts the mushroom-like flavor to *awamori* and it is derived from rice *koji* (Yoshizaki *et al*., 2010). Vanillin, the most studied volatile compound in

awamori, contributes to the sweet aroma and is produced by biochemical and chemical reactions (Koseki *et al*., 1996; Koseki and Iwano, 1998). Firstly, ferulic acid present in the rice cell wall is liberated by ferulic acid esterase produced by *A. luchuensis* during fermentation. Then, the ferulic acid is transferred to 4-vinylguaiacol (4-VG) by heating during distillation. Finally, 4-VG is converted to vanillin by chemical transformation via oxidation during aging. pH and alcohol concentration during aging influence the chemical transformation of ferulic acid to vanillin.

Rice-flavor *baijiu* is characteristically sweet aroma and has a clear aftertaste (Zheng and Han, 2016). Ethyl lactate and β-phenylethyl alcohol are key volatile compounds characteristic to rice-flavor *baijiu* compared to the other types of *baijiu* (Jin *et al*., 2017). However, any studies have not investigated flavors specific to rice-flavor *baijiu* by the comparison with *awamori* and *kome-shochu*, yet. Okinawa prefecture in Japan, the main production centre of *awamori*, and Guangdong and Guangxi provinces in China, the main production centres of rice-flavor *baijiu*, are geographically close. Therefore, rice-flavor *baijiu* is important for understanding the historical and technical relationships between Chinese and Japanese distilled liquors. A knowledge of the chemical and flavor profile of rice-flavor *baijiu* will help us to understand the similarities and/or differences between Chinese and Japanese distilled liquors.

This study aimed to determine the characteristic flavor compounds of commercially

available rice-flavor *baijiu* comparing it with *awamori* and *kome-shochu.* First, we measured the concentrations of alcohol, sugar, amino acids, and organic acids by high-performance liquid chromatography (HPLC) in rice-flavor *baijiu*. Next, we measured and compared the concentration of volatile compounds in rice-flavor *bajiu* by analysing gas-chromatography mass spectrometer (GC-MS) with those in *awamori* and *kome-shochu*. Finally, we obtained the characteristic flavor compounds in rice-flavor *baijiu* by principal component analysis derived from the quantitative results of volatile compounds of rice-flavor *baijiu*, *awamori*, and *kome-shochu.*

2-2. Materials and methods

2-2-1. Liquor samples and chemicals

We purchased 15 different commercial rice-flavor *baijiu* from a local market in China (Table 2-1) on the basis of the company, place of production and sales price range. In addition, we purchased 6 and 5 different commercial *awamori* and *kome-shochu*, respectively, from a local market in Japan. The chemicals we used were acquired from Sigma-Aldrich (Steinheim, Germany), FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan), and Nacalai Tesque, Inc. (Kyoto, Japan).

2-2-2. Total and volatile acidity of liquor samples

Total and volatile acidity values were determined following the official methods of the National Tax Administration Agency, Japan (Brewing Society of Japan, 2006). Total acidity was measured as titratable acidity. Briefly, 10 mL of each liquor sample was titrated against 0.1 N sodium hydroxide, with the indicator containing bromothymol blue and neutral red, until it turned to light green. Volatile acidity was determined by titration of each liquor sample after distillation. Briefly, 100 mL of each liquor sample was distilled using a small distillation apparatus. Approximately 70 ml of the distillate was collected, and the volume was adjusted to 100 mL with deionised water. Further, 10 mL of this solution was titrated with phenolphthalein against 0.01 N sodium hydroxide until it turned light pink. Total acidity and volatile acidity were represented in the titration volume of 0.1 and 0.01 N sodium hydroxide, respectively.

2-2-3. Quantification of saccharides

Saccharides were quantified by injecting 10 μL of each liquor sample into a Prominence high-performance liquid chromatography (HPLC) system (Shimadzu Corp., Kyoto, Japan) under the following conditions: LC-20AD pump (Shimadzu

Corp.); i.d. 4 × 250 mm Cosmosil Sugar-D column (Nacalai Tesque, Inc., Kyoto, Japan); mobile phase, acetonitrile:water (3:1); flow rate, 1.0 mL/min; and an RID-10A refractive index detector (Shimadzu Corp.) (Okutsu *et al*., 2012). Standard curves were constructed using linear regression of the analyte peak areas versus the known concentrations of each saccharide.

2-2-4. Analysis of organic acids

Organic acids were identified using the Prominence HPLC system (Shimadzu Corp.) and a CDD-10A VP conductivity detector (Shimadzu Corp.) (Rahayu *et al*., 2017). Organic acid separation was achieved using an i.d. 8 × 300 mm Shim-pack SCR-102H HPLC column (Shimadzu Corp.) at 50ºC using 4 mM *p*-toluenesulfonic acid monohydrate as a mobile phase at a flow rate of 0.8 mL/min. A mixture of 4 mM *p*-toluenesulfonic acid monohydrate, 16 mM bis-Tris and 80 μM ethylenediaminetetraacetic acid (EDTA) served as a post–column reaction solution at a flow rate of 0.8 mL/min. Standard curves were constructed using linear regression of the analyte peak areas versus known concentrations of each organic acid.

2-2-5. Analysis of amino acids

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 derivatisation method (Rahayu *et al*., 2017). Amino acid separation was achieved using a i.d. 6 × 100 mm Shimadzu Shim-pack Amino-Na column (Shimadzu Corp.) at 60ºC at 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 the excitation/emission wavelength pair of 350/450 nm, and the reaction reagents were taken from an amino acid reaction kit (Shimadzu Corp.) and maintained at a flow rate of 0.2 mL/min.

2-2-6. GC-MS with stir bar sorptive extraction

The liquor samples were diluted to 25% (v/v) alcohol by adding deionised water, 10 mL of each liquor sample was transferred to a sample vial, and a 15 mm stir bar coated with 0.5 mm polydimethylsiloxane was added (Twister, Gerstel K.K., Japan) (Rahayu *et al*., 2017). The sample was stirred on a magnetic stirrer at 1,200 rpm for 1 h at room temperature. The stir bar was removed, washed with deionised water, dried

with a tissue and placed into a glass insert. Volatile compounds were desorbed from the stir bar using the following temperature programme of the Gerstel TDS3 and Gerstel CIS4 thermal desorption system (Gerstel K.K.): 20ºC for 1 min; and 60ºC per min to 260ºC (hold for 1 min). In the meanwhile, a Gerstel CIS4 cryotrap (Gerstel K.K.) was set to −150ºC to cryofocus. After the desorptive programme was completed, the cryotrap was heated to inject the volatile compounds into a gas chromatography (GC) analytical column: −150ºC for 1 min and 12ºC per second to 270ºC (hold for 2 min). The gas chromatography–mass spectrometry (GC-MS) system was equipped with a i.d. 0.25 mm × 60 m Inner Pure-WAX column with a 0.25 μm film thickness (GL Sciences Inc., Tokyo, Japan). Analyses were carried out with helium as a carrier gas at a flow rate of 1.0 mL/min using the following temperature programme: 40ºC for 5 min; and 3ºC per min to 240ºC. The retention index (RI) was determined via sample injection with a series of straight-chain alkanes (C5–C24) (SUPELCO Analytical, PA, USA). Identification of volatile compounds was confirmed by comparing their mass spectra with the NIST05a mass spectral database and the RI values in the AromaOffice database (Nishikawa Keisoku Co., Ltd., Tokyo, Japan). Standard curves were produced from a pure authentic reagent in 25% (w/w) ethanol solution. The absolute calibration curve was drawn from peak area using selected ion (m/z). The analysis was repeated three times for each sample.

2-2-7. Data analysis

To identify which volatile compounds are likely to contribute most to the different characteristics of the liquor samples used in this study, principal component analysis (PCA) was performed using Ekuseru-Toukei 2008 statistical software (Social Survey Research Information Co., Ltd., Tokyo, Japan).

2-3. Results and discussion

2-3-1. Chemical compositions and properties

The description of 15 rice-flavor *baijiu* samples selected for this study, including details regarding their manufacturers, price range and aging periods are shown in Table 2-1. Guangdong and Guangxi provinces are famous brewing districts and the main production centers of rice-flavor *baijiu.* Therefore, 13 samples were selected from these regions, while the remaining two samples selected are made by a company in Liaoning province, northern China. Okinawa prefecture and Kumamoto prefecture are famous brewing districts in Japan and the main production centers of *awamori* and *kome-shochu*, respectively.

Of the 15 rice-flavor *baijiu* samples, two samples (S14 and S15) showed a strong dark-brown color, and one sample (S5) showed a yellow color, indicating that some rice-flavor *baijiu* samples are stored in barrels. Sugar was detected by HPLC in five rice-flavor *baijiu* samples (S3, S4, S5, S14 and S15) (Table 2-2). At least 95% of all sugars were glucose (data not shown). S14 and S15 contained a high glucose level of >60 mg/mL. S14 and S15 are samples made by the same company. Therefore, S14 and S15 samples indicated that a flavoring agent such as caramel might be added to these liquor samples. Amino acids were detected in eight samples, of which five demonstrated a low level of amino acids; the remaining three samples (S5, S14 and S15) had a high level of amino acids. These amino acids may also be derived from a flavoring agent, in the same manner as glucose.

Rice-flavor *baijiu* showed higher total acidity compared to *awamori* and *kome-shochu*, with a wide total acidity range among the 15 samples. Sample S15 showed the highest total acidity, which corresponds to 8.4-fold higher than that of the lowest sample S8 (Table 2-2). On the other hand, volatile acidity was markedly lower than total acidity, with rice-flavor *baijiu* having slightly higher volatile acidity compared to *awamori* and *kome-shochu*. However, we found a large difference between total acidity and volatile acidity in rice-flavor *baijiu*. Therefore, acid compositions were analysed by HPLC (Table 2-2). All the rice-flavor *baijiu* samples mainly contained lactic acid and acetic

acid. Other acids were detected in S5, S14 and S15, but their levels were very low (data not shown). Sample S15 contained the highest levels of lactic acid, which corresponds to14-fold higher than that of the lowest sample S8. While Sample S14 showed the highest levels of acetic acid, which corresponds to 4-fold higher than that of the lowest sample S9. The levels of lactic acid correlated with the total acidity levels in all rice-flavor *baijiu* samples, indicating that a high lactic acid level results in high total acidity in rice-flavor *baijiu.* Lactic acid is a non-volatile acid, and it is generally difficult to detected in distilled liquors. Thus, these results imply that the addition of lactic acid is general technique throughout rice-flavor *baijiu* and the addition of sugar is restrictive. The concentrations of lactic acid and glucose detected in rice-flavor *baijiu* were higher than that of amino acid. Amino acid detected in five samples might come from the reagents of flavoring and lactic acid as impurity.

In China, there is a national standard for rice-flavor *baijiu* developed by the Standardisation Administration of the People's Republic of China (SAC) (GB/T 10781.3-2006) (Yu, 2016). According to this standard, the quality level is prescribed according to sensory evaluation, total acidity, total ester content, ethyl lactate content, β-phenylethyl alcohol content and solid content. A >0.3 g/L (= 5 mM acetic acid) total acid content equivalent to acetic acid means a high quality level. Therefore, a high level of acetic acid is one of the characteristics of rice-flavor *baijiu.*

2-3-2. Quantitation by GC-MS

GC-MS with stir bar sorptive extraction identified and quantified 34 compounds from mass spectra and the RI: 5 alcohols; 3 acids; 22 esters; 2 furans; 1 aldehyde; and 1 sulfuric compound (Table 2-3). Rice-flavor *baijiu* contained a wide concentration range of 18 volatile compounds with an odor activity value (OAV) of >1 (Table 2-4). Both *awamori* and *kome-shochu* contained 13 compounds with OAV of >1. Of these compounds, 11 showed >3-fold OAV in rice-flavor *baijiu* compared to OAV of *awamori* and *kome-shochu*: ethyl isobutyrate; ethyl isovalerate; ethyl lactate; ethyl caproate; ethyl laurate; ethyl myristate; ethyl palmitate; ethyl linoleate; 2-pentyl furan; 2-nonenal; and dimethyl trisulfide.

Ethyl isobutyrate, ethyl isovalerate and ethyl lactate levels depend on the concentration of the organic acids isobutyric acid, isovaleric acid and lactic acid, respectively, in the fermented mash (Shen, 2003; Rahayu *et al*., 2017). Ethyl lactate is the common and important compound in Chinese liquor. Esterification of ethanol and lactic acid in Chinese liquor are carried out during fermentation (Cheng *et al*., 2018). Lactic acid in the rice-flavor *baijiu* mash is mainly produced by mold and lactic acid bacteria in *xiaoqu* (Yin *et al*., 2019), while citric acid is the main organic acid in the *awamori* and *kome-shochu* mashes because *koji* molds (*A. luchuensis* and *A. luchuensis*

mut. *kawachii*) used in *shochu* making mainly produce and secrete a citric acid (Kadooka *et al*., 2019). Therefore, the lactic acid content of the *awamori* and *kome-shochu* mashes is not high, about 10 times lower than that of rice-flavor *baijiu* (Zhao, 2019). These findings suggest that ethyl lactate is a characteristic compound in rice-flavor *baijiu* compared to *awamori* and *kome-shochu*.

Fatty acid ethyl esters such as ethyl caproate, ethyl laurate, ethyl myristate, ethyl palmitate and ethyl linoleate were found abundant in rice-flavor *baijiu*. The genus *Rhizopus* is recognized as a good lipase producer, and its lipase is used in many biotechnological applications (Yu, Xu, and Xiao, 2016). On the other hand, *A. oryzae*, a popular *koji* mold, does not produce a large amount of lipase in solid culture (Ohnishi *et al*., 1994). Therefore, it is suggested that long-chain fatty acid levels in the rice-flavor *baijiu* mash are higher compared to *awamori* and *kome-shochu*, and these ethyl esters are also produced by yeast. Moreover, all rice-flavor *baijiu* samples in this study had a higher alcohol level compared to *awamori* and *kome-shochu* (Table 2-1). Therefore, long-chain fatty acid ethyl esters are able to solve and found in large quantities in Chinese liquor.

Ethyl caproate is one of the important flavor compounds in Chinese liquor (Shen, 2003). It is generally produced by yeast. Although, yeast is a common microorganism used for rice-flavor *baijiu*, *awamori* and *kome-shochu*, yeast strain has a greater

influence than fermentations condition such as aeration and fermentation temperature on the formation of ethyl caproate (Piendl and Geiger, 1980). Ethyl caproate is synthesized via two pathways by yeasts: from caproic acid and ethanol by esterase and from caproyl-CoA and ethanol by alcohol acyltransferase (Liu *et al*., 2004). The synthesis of ethyl caproate in brewing is limited the abundance of caproic acid in the fermentation mash. Therefore, the difference of yeast strain and fermentation conditions among rice-flavor *baijiu* and *awamori* and *kome-shochu* might affect on the level of ethyl caproate in each liquor. Furthermore, caproic acid–producing bacteria, such as *Clostridium kluyveri* is well known to contribute to the production of ethyl caproate in Chinese liquors (Hu *et al*., 2015). Therefore, caproic acid–producing bacteria might be the reason for the large amount of ethyl caproate in rice-flavor *baijiu.* However, these microbes in rice-flavor *baijiu* manufacturing have not yet been researched. Future studies are required in order to reveal the relationship between minor microbes and the flavor of rice-flavor *baijiu.*

β-Phenylethyl alcohol has also been previously reported as a characteristic compound in rice-flavor *baijiu* (Jin *et al*., 2017). β-Phenylethyl alcohol has a rose-like odor and is found in important aroma compounds in various alcoholic beverages (Lilly *et al*., 2006). In this study, level of β-phenylethyl alcohol in rice-flavor *baijiu* and *awamori* was almost at the same, but was >2.5-fold by comparison with *kome-shochu*.

β-Phenylethyl alcohol is produced from yeast by two pathways during fermentation: degradation of phenylalanine to alcohol in the Ehrlich pathway and from phenylpyruvate during phenylalanine synthesis from carbohydrates (Äyräpää, 1965). The high alcohol level in β-phenylethyl alcohol is controlled by the amino acid level of the liquor mash via either pathway.

Kome-shochu manufacturing is different from that of rice-flavor *baijiu* and *awamori* (Fig. 2-1). Rice-flavor *baijiu* and *awamori* are produced from saccharified rice or rice *koji*, while *kome-shochu* is produced from steamed rice added to the liquor mash after five days of fermentation. The saccharified rice and rice *koji* contain various protease enzymes from the mold in the fermentation starter (Long *et al*., 2013; Machida, 2002). We believe that enzyme activity in the rice-flavor *baijiu* and *awamori* mashes is more abundant compared to *kome-shochu*. Therefore, the amino acid level during fermentation may be different, which affects the high alcohol level of liquor.

Volatile compounds with a three-fold higher OAV in *awamori* or *kome-shochu* compared to rice-flavor *baijiu* were 1-butanol, ethyl caprylate, isoamyl acetate and phenylethyl acetate. Isoamyl acetate and phenylethyl acetate are produced in yeast cells by alcohol acetyl-transferases (AATases; EC 2.3.1.84) from alcohols and acetyl–coenzyme A (acetyl-CoA) as a substrate. AATase activity and the gene expression level of the AATase gene *ATF1* are inhibited by unsaturated fatty acids (Fujii *et al*., 1997).

The fatty acid content of the rice-flavor *baijiu* mash is high because of lipase production by *Rhizopus* sp., as previously described (Yu, Xu, and Xiao, 2016). Therefore, AATase of yeast might be more strongly inhibited in rice-flavor *baijiu* mash compared to *awamori* and *kome-shochu.*

2-3-3. PCA of volatile compounds

PCA results showed diversity among the 15 rice-flavor *baijiu* samples (Fig. 2-2), while all six *awamori* samples and most of the five *kome-shochu* samples were grouped into the same cluster. The first and second principal components (PC1 and PC2) correlated positively with the diversity of rice-flavor *baijiu* and the differences between Chinese and Japanese distilled liquors, respectively. In particular, ethyl lactate and isoamyl alcohol were important compounds distinct between rice-flavor *baijiu*, and *awamori* and *kome-shochu*. Isobutyl alcohol, β-phenylethyl alcohol, and ethyl caproate contributed to distinct types of rice-flavor *baijiu*. Rice-flavor *baijiu* types made by the same company were plotted in a closed position; S1 and S2, S5-S7, S8 and S9, S10–S12, and S14 and S15. The difference by aging period was small. Therefore, the flavor of rice-flavor *baijiu* strongly depends on the company.

2-4. Summary

This study aimed to reveal the chemical and flavor profiles of rice-flavor *bajiu* by comparing with *awamori* and *kome-shochu*, traditional Japanese liquors. Rice-flavor *baijiu* is similar to *awamori* and *kome-shochu* with regard to ingredients and the fermentation starter. Of the 15 rice-flavor *baijiu* samples, 3 had a light yellow to dark-brown color. Dark-brown samples had a high glucose and low amino acid contents. Lactic acid was detected in all rice-flavor *baijiu* samples. Compared to *awamori* and *kome-shochu*, rice-flavor *baijiu* contained more acetic acid. We identified and quantified 34 volatile compounds in rice-flavor *baijiu*. In all, 18 compounds in rice-flavor *baijiu*, 13 in *awamori* and *kome-shochu* had an odor activity value (OAV) of >1. Of these, 11 compounds showed a three-fold higher OAV in rice-flavor *baijiu* than in *awamori* and *kome-shochu*. Principal component analysis revealed that ethyl lactate is a key volatile compound that is distinct in rice-flavor *baijiu.*

TABLE 2-1. Sample informations used in this study.

	Types of liquor	Region, Country	Company	Aging periods (years)	Distillation type	Alcohol† (%, v/v)
S1	Rice-flavor *baijiu*	Guangxi, China	A	5	Atmospheric	35
S2	Rice-flavor *baijiu*	Guangxi, China	A	Non aging*	Atmospheric	30
S3	Rice-flavor *baijiu*	Guangxi, China	B	18	-	49
S4	Rice-flavor *baijiu*	Guangxi, China	C	18	-	53
S5	Rice-flavor *baijiu*	Guangxi, China	D	5	-	49
S6	Rice-flavor *baijiu*	Guangxi, China	D	-	-	46
S7	Rice-flavor *baijiu*	Guangxi, China	D	-	-	48
S8	Rice-flavor *baijiu*	Guangdong, China	E	5	Atmospheric	48
S9	Rice-flavor *baijiu*	Guangdong, China	E	Non aging*	Atmospheric	52
S10	Rice-flavor *baijiu*	Guangdong, China	F	-	-	49
S11	Rice-flavor *baijiu*	Guangdong, China	F	10	-	54
S12	Rice-flavor *baijiu*	Guangdong, China	F	5	-	48
S13	Rice-flavor *baijiu*	Guangdong, China	G	-	-	47
S14	Rice-flavor *baijiu*	Liaoning, China	H	-	-	50
S15	Rice-flavor *baijiu*	Liaoning, China	H	-	-	54
A1	*Awamori*	Okinawa, Japan		Non aging*	Atmospheric	30
A2	*Awamori*	Okinawa, Japan		Non aging*	Mixture of vacuum and atmospheric	30
A3	*Awamori*	Okinawa, Japan		Non aging*	Vacuum	20
A4	*Awamori*	Okinawa, Japan		Non aging*	Atmospheric	30
A5	*Awamori*	Okinawa, Japan		Non aging*	Mixture of vacuum and atmospheric	30
A6	*Awamori*	Okinawa, Japan		Non aging*	Atmospheric	30
K1	*Kome-shochu*	Kumamoto, Japan		Non aging*	Vacuum	25
K2	*Kome-shochu*	Kumamoto, Japan		Non aging*	Mixture of vacuum and atmospheric	25
K3	*Kome-shochu*	Kumamoto, Japan		3~5*	Atmospheric	25
K4	*Kome-shochu*	Kumamoto, Japan		Non aging*	Vacuum	25
K5	*Kome-shochu*	Kumamoto, Japan		3*	Vacuum	25

† Alcohol content of each sample was measured in our labolatory.

Awamori and kome-shochu samples are made by all different companies.

Aging periods were described what was entered into the package. "-" means we don't have any information about aging time.

*The information of aging time was confirmed with each company directly.

TABLE 2-2. Analysis of spirits

	Rice-flavor *baijiu*			*Awamori*			*Kome-shochu*		
	mean	max	min	mean	max	min	mean	max	min
Total acidity	13.8	37	4.4	0.71	1.45	0.10	0.33	0.91	0.09
Volatile acidity	1.70	2.8	0.45	-	-	-	-	-	-
Glucose (mM)	62	386	0	-	-	-	-	-	-
Amino acid (μM)	296	1978	0.9	-	-	-	-	-	-
Lactic acid (mM)	7.4	23.8	1.7	nd	nd	nd	nd	nd	nd
Acetic acid (mM)	5.0	10.5	2.6	0.85	1.70	nd	0.55	1.49	nd

nd, not detected. "-" means not to measure. Fifteen rice-flavor *baijiu* samples, six *awamori* samples, and five *kome-shochu* samples were used.

TABLE 2-3. Volatile compounds in rice-flavor *baijiu*, *awamori*, and *kome-shochu* detected by GC-MS.

	Compounds	Odor description	RI	Identification	CAS No	Quantifi-
Alcohol	Isobutyl alcohol	Fusel, alcohol	1092	MS, RI, STD	78-83-1	43
	Isoamyl alcohol	Alcohol, harsh, bitter	1209	MS, RI, STD	123-51-3	55
	1-Butanol	Medicinal, alcohol	1143	MS, RI, STD	71-36-3	56
	β-Phenylethyl alcohol	Floral, rose	1872	MS, RI, STD	60-12-8	91
	1-Hexanol	Veg*et al*, herbaceous	1339	MS, RI, STD	111-27-3	56
Acid	Octanoic acid	Sweet, chees	2026	MS, RI, STD	124-07-2	60
	Decanoic acid	Fatty, unpleasant	2233	MS, RI, STD	334-48-5	73
	Dodecanoic acid	Dry, m*et al*lic, laurel oil	2450*	MS, RI, STD	143-07-7	73
Ester	Ethyl isobutyrate	Fruity, strawberry	945	MS, RI, STD	97-62-1	43
	Ethyl isovalerate	Fruity	1060	MS, RI, STD	108-64-5	88
	Ethyl lactate	Fruity, lactic, raspberry	1354	MS, RI, STD	97-64-3	45
	Ethyl caproate	Fruity, floral	1221	MS, RI, STD	123-66-0	88
	Ethyl caprylate	Pineapple, pear, floral	1419	MS, RI, STD	106-32-1	88
	Ethyl caprate	Fruity, fatty, solvent	1620	MS, RI, STD	110-38-3	88
	Ethyl laurate	Sweet, floral, fruity, cream	1830	MS, RI, STD	106-33-2	88
	Ethyl myristate	-	2030	MS, RI, STD	124-06-1	88
	Ethyl palmitate	Fatty, rancid, fruity, sweet	2235	MS, RI, STD	628-97-7	88
	Ethyl stearate	-	2442*	MS, RI, STD	111-61-5	88
	Ethyl oleate	-	2460*	MS, RI, STD	111-62-6	55
	Ethyl linoleate	-	2505*	MS, RI, STD	544-35-4	67
	Ethyl salicylate	-	1774	MS, RI, STD	118-61-6	120
	Ethyl benzoate	Fruity	1635	MS, RI, STD	93-89-0	105
	Ethyl phenylacetate	Rose, honey	1753	MS, RI, STD	101-97-3	91
	Isoamyl acetate	Banana	1116	MS, RI, STD	123-92-2	43
	Isoamyl caproate	Pineapple, cheese	1451	MS, RI, STD	2198-61-0	70
	Isoamyl caprylate	Sweet, light fruity, cheese, cream	1645	MS, RI, STD	2035-99-6	70
	Isobutyl caprylate	-	1541	MS, RI, STD	5461-06-3	127
	Phenylethyl acetate	Floral, rose	1783	MS, RI, STD	103-45-7	104
	Phenylethyl butyrate	Fruity	1793	MS, RI, STD	103-52-6	104
	Phenylethyl octanoate	-	2345	MS, RI, STD	5457-70-5	104
Furan	2-Pentyl furan	Green bean-like	1222	MS, RI, STD	3777-69-3	81
	Furfural	Bread, sweet	1431	MS, RI, STD	000098-01-1	95
Aldehyde	2-Nonenal	Green	1512	MS, RI, STD	2463-53-8	70
Sulfric compound	Dimethyl trisulfide	Cooked onion	1355	MS, RI, STD	3658-80-8	126

*Because RI was slightly out of the range, these RIs were estimated by adapting the conversion formula from retention time to RI prepared.

TABLE 2-4. The concentration (μg/L) and odor-active values of volatile compounds in rice-flavor *baijiu*, *awamori*, and *kome-shochu*.

		Rice-flavor *baijiu*			*Awamori*			*Kome-shochu*		
		Mean	Max	Min	Mean	Max	Min	Mean	Max	Min
Alcohol	Isobutyl alcohol	190,194	564,045	27,663	126,280	210,516	79,231	183,592	422,564	66,233
	Isoamyl alcohol	323,628	634,175	38,761	337,228	393,797	251,755	257,265	351,014	142,027
	1-Butanol	3,873	9,330	nd	5,160	8,931	2,152	9,645	23,666	3,988
	β-Phenylethyl Alcohol	80,707	456,898	18,760	72,604	108,734	40,455	31,901	39,336	24,649
	1-Hexanol	315	1,081	nd	30	53	nd	97	185	nd
Acid	Octanoic acid	1,168	3,321	43	1,188	2,820	454	381	1,643	19
	Decanoic acid	1,339	2,867	54	777	1,529	342	295	1,183	nd
	Dodecanoic acid	436	970	12	168	352	54	13	21	4.7
Ester	Ethyl isobutyrate	1,366	3,946	191	242	613	101	72	207	tr
	Ethyl isovalerate	48	103	15	19	35	10	5.0	13	nd
	Ethyl lactate	379,079	641,297	93,568	4,475	7,426	nd	nd	nd	nd
	Ethyl caproate	24,536	202,446	21	550	917	183	7,343	35,081	240
	Ethyl caprylate	2,221	5,857	3.0	1,270	2,133	60	6,635	31,048	297
	Ethyl caprate	2,891	8,431	2.2	320	804	7.1	1,144	5,356	54
	Ethyl laurate	538	1,596	Tr	11	18	Tr	16	59	1.3
	Ethyl myristate	1,159	2,765	Tr	0.6	1.2	nd	7.4	16	Tr
	Ethyl palmitate	15,623	171,874	Tr	Tr	Tr	nd	5.0	14	Tr
	Ethyl stearate	24	289	nd	nd	nd	nd	1.2	6.0	nd
	Ethyl oleate	359	2,011	nd	14	84	nd	1.5	6.2	nd
	Ethyl linoleate	4,630	19,391	nd	0.8	1.1	nd	1.0	1.7	0.9
	Ethyl salicylate	0.8	3.1	nd	0.04	0.22	nd	0.8	3.1	nd
	Ethyl benzoate	15	37	nd	1.6	1.6	Tr	1.6	3.0	nd
	Ethyl phenylacetate	5.7	10	2.1	3.4	4.7	1.9	1.6	3.8	nd
	Isoamyl acetate	997	3,273	nd	2,955	5,151	1,774	3,672	7,646	78
	Isoamyl caproate	0.6	1.5	nd	0.36	0.43	Tr	2.9	13	nd
	Isoamyl caprylate	10	25	nd	2.8	3.6	Tr	2.7	10	0.7
	Isobutyl caprylate	1.6	3.6	nd	0.4	0.5	Tr	0.6	2.1	nd
	Phenethyl acetate	322	846	15	1,364	1,422	655	982	2,035	573
	Phenylethyl butyrate	2.3	2.9	Tr	1.3	1.5	1.2	1.0	1.0	Tr
	Phenylethyl octanoate	1.7	7.0	nd	1.0	1.4	nd	1.0	4.0	nd
Furan	2-Pentyl furan	8.0	26	nd	0.2	1.4	Tr	1.5	2.3	nd
	Furfural	325	2,437	nd	705	1,747	nd	nd	nd	nd
Aldehyde	2-Nonenal	20	94	nd	3.3	8.8	nd	3.1	15	nd
Sulfric compound	Dimethyl trisulfide	4.3	10	nd	1.5	2.2	nd	nd	nd	nd

Method of indentification: MS, mass spectrum comparison using NIST05a library; RI: retention index in agreement with literature value; STD, confirmed by authentic standards. nd, not detected. tr, trace. Fifteen rice-flavor *baijiu* samples, six *awamori* samples, and five *kome-shochu* samples were used.

TABLE 2-4. Continued.

			OAV			
		Odor threshold (μg/L)	Rice-flavor *baijiu*	*Awamori*	*Kome-shochu*	Reference
Alcohol	Isobutyl alcohol	40,000	4.8	3.2	4.6	1
	Isoamyl alcohol	30,000	11	11	8.6	1
	1-Butanol	5,000	1>	1.0	1.9	2
	β-Phenylethyl Alcohol	10,000	8.1	7.3	3.2	1
	1-Hexanol	8,000	1>	1>	1>	1
Acid	Octanoic acid	15,000	1>	1>	1>	2
	Decanoic acid	15,000	1>	1>	1>	1
	Dodecanoic acid	7,200	1>	1>	1>	2
Ester	Ethyl isobutyrate	15	91	16	4.8	1
	Ethyl isovalerate	3	16	6.4	1.7	1
	Ethyl lactate	14,000	27	1>	1>	2
	Ethyl caproate	5	4,910	110	1,470	1
	Ethyl caprylate	2	1,110	635	3,320	1
	Ethyl caprate	200	14	1.6	5.7	3
	Ethyl laurate	500	1.1	1>	1>	4
	Ethyl myristate	500	2.3	1>	1>	4
	Ethyl palmitate	14,000	1.1	1>	1>	2
	Ethyl stearate	500	1>	1>	1>	2
	Ethyl oleate	870	1>	1>	1>	2
	Ethyl linoleate	450	10	1>	1>	2
	Ethyl salicylate	-	-	-	-	-
	Ethyl benzoate	575	1>	1>	1>	3
	Ethyl phenylacetate	100	1>	1>	1>	5
	Isoamyl acetate	30	33	99	122	1
	Isoamyl caproate	1,400	1>	1>	1>	2
	Isoamyl caprylate	125	1>	1>	1>	3
	Isobutyl caprylate	800	1>	1>	1>	6
	Phenethyl acetate	250	1.3	5.5	3.9	1
	Phenylethyl butyrate	961	1>	1>	1>	7
	Phenylethyl octanoate	-	-	-	-	-
Furan	2-Pentyl furan	1	8.0	1>	1.5	8
	Furfural	14,100	1>	1>	1>	3
Aldehyde	2-Nonenal	0.08	255	41	39	8
Sulfric compound	Dimethyl trisulfide	0.2	21	7.6	1>	1

1. Guth, 1997; 2. Salo *et al*., 1972; 3. Ferreira *et al*. 2000; 4. Zea *et al*., 2001; 5. Isogai *et al*., 2005; 6. Li *et al*., 2008; 7. Wang *et al*., 2014b; 8. Buttery, *et al*., 1988. Fifteen rice-flavor *baijiu* samples, six *awamori* samples, and five *kome-shochu* samples were used.

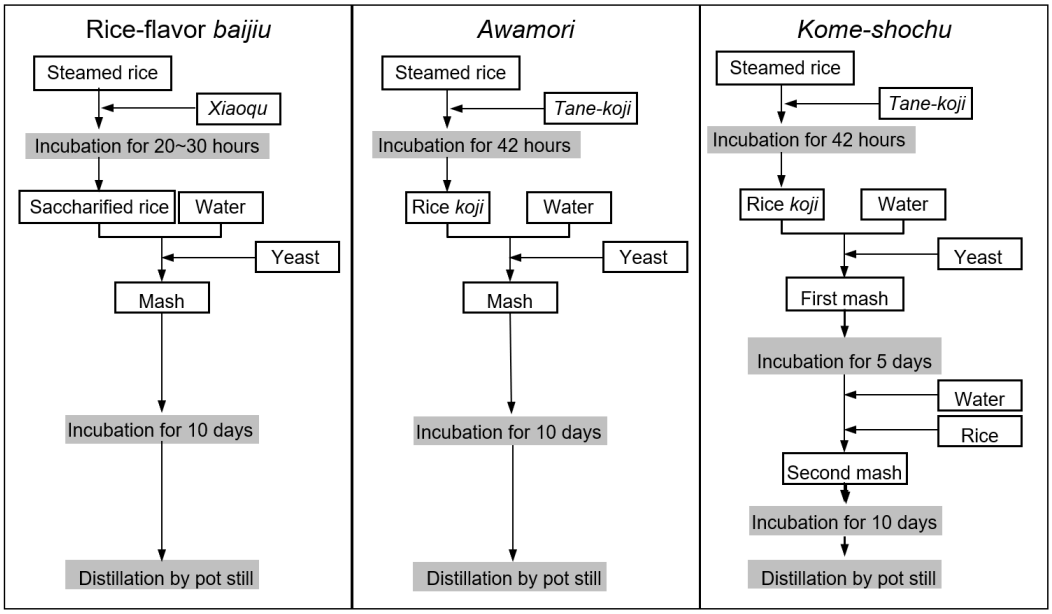

FIG. 2-1. Schematic diagram of manufacturing of rice-flavor *baijiu*, *awamori* and *kome-shochu*.

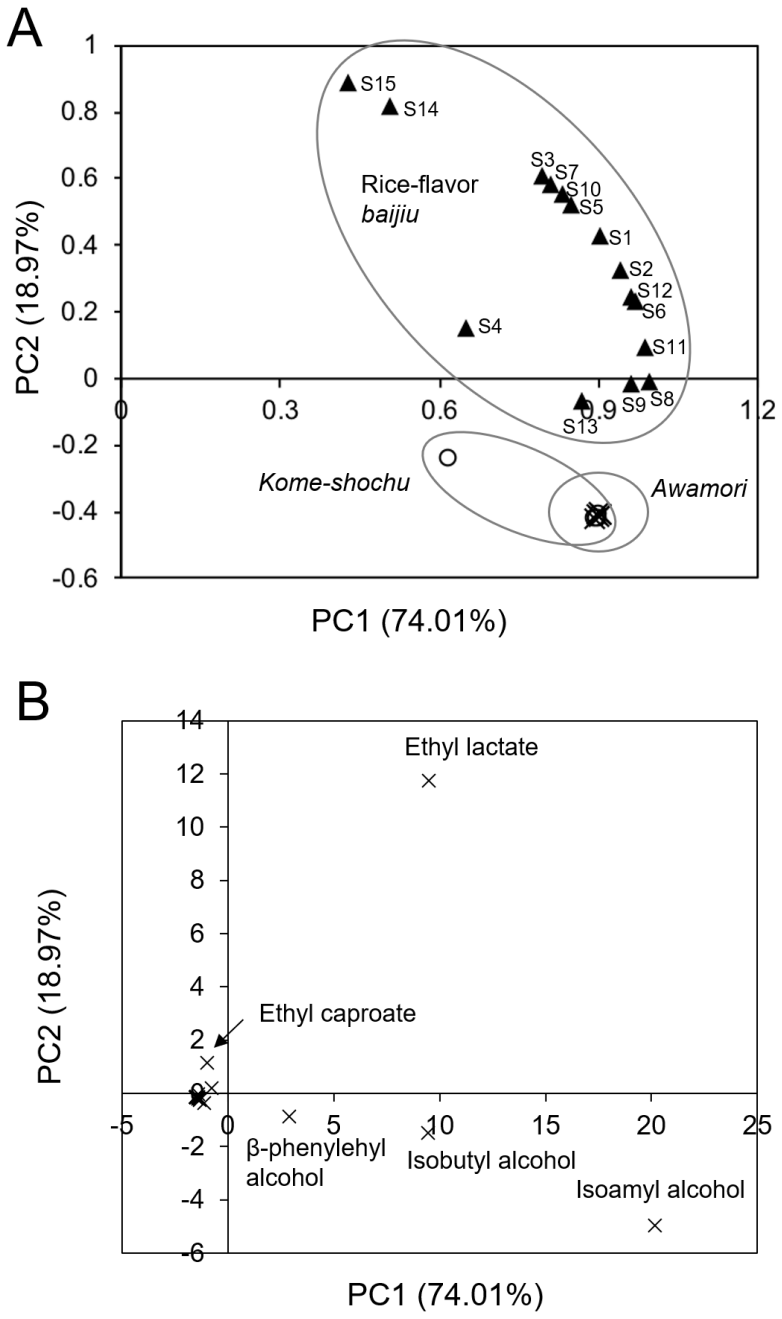

FIG. 2-2. Principal component analysis (PCA) biplot of the concentrations of volatile compounds in the liquor samples used in the study. A, scores plot of PCA. B. loading plot of PCA. In panel A the filled triangles represent rice-flavor *baijiu* samples, the crosses represent *awamori* samples, the open circles represent *kome-shochu* samples.

Chapter 3. Manufactural impact of the solid-state saccharification process in rice-flavor *baijiu* production

3-1. Introduction

Baijiu is a traditional liquor in China. It is classified into four major flavor types, including sauce-, strong-, light-, and rice-flavor. The different flavor types are associated with different regions of China and are obtained by applying different production processes, raw materials, and fermentation starters (*jiuqu*) (Liu and Sun, 2018). *Jiuqu* are prepared by culturing microorganisms for brewing from cereals; therefore, they contain numerous enzymes produced by microorganisms. The enzymes decompose starch into fermentable sugars, which facilitates fermentation. Consequently, the effects of *jiuqu* on *baijiu* are considered similar to the effects of *koji* on *shochu* (a traditional Japanese liquor) and malt on whiskey (Zhu and Tramper, 2013). Generally, two major types of *jiuqu* are used in *baijiu* production including *daqu* and *xiaoqu* (Jin *et al*., 2017).

Rice-flavor *baijiu* is popular in the southern regions of China such as Guangxi and Guangdong. It is manufactured using rice as the sole raw material and *xiaoqu*. *Xiaoqu* is usually prepared by cultivating mold in rice flour, and in some cases with yeast (Jin

et al., 2017; Gou *et al*., 2015; Zheng and Han, 2016). Consequently, rice-flavor *baijiu* is characterized by light and sweet flavors associated with rice. The process of manufacturing rice-flavor *baijiu* is as follows. Rice is the main ingredient. Raw rice is washed and soaked in water for 1 h, and then the water is drained. Subsequently, the soaked rice is steamed for 1 h, and a little amount of *xiaoqu* is added to the steamed rice, then the products are mixed well. The mixture is incubated for 20–30 hours. During incubation, the starch in the steamed rice is decomposed into sugars by enzymes from *xiaoqu* in the absence of free-flowing water. Therefore, such an incubation process is referred to as solid-state saccharification. After saccharification, water and yeast used in brewing are added and the mixture was fermented in a liquid-state for 6–8 days. Finally, the fermented mash is distilled (Wang, 2003b, 2003c, and 2003d; Hou and Xiao, 1999; Zheng, 2003; Huang *et al*., 2013). In brief, rice-flavor *baijiu* is produced via solid-state saccharification, liquid-state fermentation, and distillation.

The other three types of *baijiu* flavors in China use *daqu* as the fermentation starter and are produced via solid-state saccharification, fermentation, and distillation. On the other hand, Japanese *shochu* production uses *koji* as the fermentation starter followed by liquid-state fermentation and distillation processes. Therefore, the process of manufacturing rice-flavor *baijiu* seems to integrate the production processes of Chinese and Japanese liquor as described above. In addition, its manufacturing processes are

more similar to those of *shochu*. Unlike *shochu,* rice-flavor *baijiu* employs a solid-state saccharification process, which converts 70%–85% of the starch in rice into fermentable sugars (Zheng, 2003). The solid-state saccharification process is a characteristic method applied in the manufacturing of Chinese *baijiu*. However, no study has investigated the role of the solid-state saccharification process in the preparation of rice-flavor *baijiu*. An understanding of the role of the solid-state saccharification would facilitate the maintenance of quality in *baijiu* preparation. In the present study, we investigated the role of the solid-state saccharification process in the manufacturing rice-flavor *baijiu* by evaluating changes in biochemical properties and microbial communities before and after solid-state saccharification. Furthermore, we discuss the appropriate saccharification conditions that could facilitate the stable production of rice-flavor *baiji*u.

3-2. Materials and Methods

3-2-1. Materials, chemicals, and strain

The chemicals used for analysis 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 used was Kagoshima-5, supplied by the Kagoshima Prefectural Brewing Association (Kagoshima, Japan) (Takamine *et al*., 1994). Polished rice was purchased from Hombo Shoten Co., Ltd. (Kagoshima, Japan).

3-2-2. Solid-state saccharification

The rice was steamed twice to obtain the original water content 60%–63% (Wang, 2003c; Hou and Xiao, 1999; Zheng, 2003). The rice was first soaked in water, drained off, and steamed for 1 h, and then the required volume of hot water was added until the water content reached 60%. The rice was finally steamed for 1 h again. After that, *xiaoqu,* at 1% of the weight of the raw rice, was mixed thoroughly with the steamed rice. The mixture was incubated at 35ºC for 24 h. The obtained product was considered the fundamental solid-state saccharification in the present study. To determine the optimal saccharification temperature, the incubation temperature was adjusted to 25ºC, 30ºC, 38ºC, and 40ºC.

3-2-3. Alcohol fermentation monitoring

We prepare two fermentation mashes. One mash was prepared by using the general method of rice-flavor *baijiu*. The steamed rice (corresponding to 200 g of raw rice) was saccharified according the process described above. A total 235 ml of water and 5 ml of yeast seed culture (2.7×10^8 cells/ml) were added to the saccharified sample, which was then incubated at 30ºC for 7–11 days for alcohol fermentation. Another fermentation was prepared without the solid-state saccharification process. Alcohol fermentation was monitored by measuring the amount of CO_2 gas generated. The CO_2 gas was completely released after the sample was uniformly stirred, reducing the weight of the sample. Samples were weighed after being stirred at the same time each day. The daily decrease in weight from the initial fermentation day was used 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 alcohol fermentation.

3-2-4. Sugar analysis

After saccharification the sample (corresponding to 50 g of raw rice) was added to 250 ml of deionized water and homogenized in ice. The homogenate was used to

measure total sugar content. The homogenate was centrifuged at 10,000 ×*g* for 15 minutes at 4ºC, and then the supernatant was used to measure glucose and reducing sugar contents. The glucose content was measured via the Mutarotase-GOD method using a Glucose CII-Wako kit (FUJIFILM Wako Pure Chemical Co., Osaka, Japan). The reducing sugar content was determined via a Somogyi-Nelson assay (Nelson, 1944; Somogyi, 1952). The amount of reducing sugar in the sample was obtained from the calibration curve of the glucose concentration by measuring the absorbance at 540 nm. Total sugar content was measured via the Somogyi method after acid hydrolysis (Joslyn,1945; Ebine and Nakajima, 1955). The carbohydrate hydrolysate of the sample was determined as glucose using a simple and accurate modified Somogyi method (Kobayashi and Tabuchi, 1954).

3-2-5. Enzyme assays

Xiaoqu and the samples after saccharification were used 20.0 g and 20.8 g, respectively. The sample was suspended in the 30 ml of extraction buffer [100 mM acetate buffer (pH 5.0) contained 0.5% (w/v) NaCl] and maintained at 4ºC overnight. The suspension was centrifuged at 13,700 ×*g* for 5 minutes, and the resulting supernatant was collected. The precipitate was re-suspended with extraction buffer, and

the supernatant was collected by centrifugation again. This operation was repeated twice. Finally, the three supernatants were combined and made up to 100 ml with extraction buffer. A 10 ml extract was then dialyzed against a 10 mM acetate buffer (pH 5.0) at 4ºC overnight, then it was made up to 20 ml with deionized water. The dialyzed solution was used as a crude enzyme to measure the activity.

The α-amylase, protease and saccharification activities were measured by the Official Methods of the National Tax Administration Agency, Japan (Brewing Society of Japan, 2006) with some modifications. The enzyme reaction was carried out at 35ºC and pH 3.5. One unit of α-amylase activity was defined as the amount of enzyme which hydrolyze 1 ml of 1% (w/v) starch per 30 minutes at 35ºC. Two milliliters of citrate buffer (20 mM, pH 3.5) containing 1% (w/v) soluble starch was preincubated at 35ºC for 5 minutes. Then, 0.1 ml of crude enzyme was added and allowed to react at 35ºC. Afterward, 0.1 ml of the reaction mixture was added to 10 ml of 0.25 mM iodine solution containing 0.5% (w/v) HCl and incubated at 25ºC for 10 minutes. The transmittance of the reaction mixture was recorded at 670 nm (T%). The transmittance of the mixture without crude enzyme was also obtained (T_0%). The α-amylase activity was calculated by the formula [$12.75 \times (T\%-T_0\%)/t$] (t = reaction time (min)) (Shinoki *et al*., 1985).

One unit of protease activity was defined as the amount of enzyme required to

liberate 1 μg of tyrosine from casein in 60 minutes at 35ºC. The mixture of 1.5 ml casein solution [2% (w/v) casein in 20 mM citrate buffer, pH 3.5] and 1 ml citrate buffer (50 mM, pH 3.5) was preincubated at 35ºC for 5 minutes. The crude enzyme solution (0.5 ml) from *xiaoqu* was added to the casein mixture and reacted for 10 min at 35ºC. The crude enzyme solution (1 ml) from the sample after saccharification was added to the casein mixture and reacted for 280 min at 35ºC. The reactions were terminated by addition of 3 ml of 0.4 M trichloroacetic acid. The mixtures were centrifuged at 13,700 ×*g* for 5 minutes. One milliliter of supernatant was mixed with 5 ml of 0.4 M Na_2CO_3 and 1 ml of Folin-Ciocalteu's phenol reagent. After incubation at 40ºC for 30 minutes, the absorbance at 660 nm was measured. The amount of tyrosine liberated was determined from the absorbance standard curve of tyrosine.

One unit of saccharification activity was defined as the amount of enzyme which produce 1 mg of glucose per 60 minutes from starch at 35ºC. One milliliter of citrate buffer (20 mM, pH 3.5) containing 2% (w/v) soluble starch was preincubated at 35ºC for 5 minutes. Then, 0.1 ml of crude enzyme was added to react at 35ºC for 10 or 60 minutes and terminated by the addition of 0.1 ml of 1 N NaOH. After incubation for 30 minutes at 25ºC, 0.1 ml of 1 N HCl was added to neutralize the reaction mixture. The amount of glucose produced was analyzed by using the Glucose CII-Wako kit.

3-2-6. Organic acid content analysis

The samples before and after solid-state saccharification were extracted with deionized water in the same manner as that in the enzyme assay. The extract was filtered through a membrane filter with a 0.45-μm pore size. Organic acids were identified using a high-performance liquid chromatograph (HPLC) (Prominence HPLC system, Shimadzu Corp., Kyoto, Japan) and an electric conductivity detector (CDD-10A VP, Shimadzu Corp.). Separation was conducted by two analytical columns (Shim-pack SCR-102H 300 mm × 8 mm *i.d.*, Shimadzu Corp.) at 50ºC using 4 mM *p*-toluenesulfonic acid as the mobile phase at a flow rate of 0.8 ml/min. The mixture of 4 mM *p*-toluenesulfonic acid, 16 mM bis-tris, and 80 μM EDTA served as a post column reaction solution at a flow rate of 0.8 ml/min.

3-2-7. Enumeration of bacterial and fungal colonies

A dilution spread-plate method was used to determine the colony-forming units (CFU). Fungi were grown on potato dextrose agar (PDA) plates containing 0.01% chloramphenicol and 0.1% Triton X-100. To observe the mold colonies, the plate was incubated at 30ºC for 24 h under aerobic condition. To observe the yeast colonies, the

plate was incubated at 30ºC for 96 h under anaerobic conditions using the Anaeropack system (Mitsubishi Gas Chemical Company Inc., Tokyo, Japan). The colonies were observed every 24 h. Bacteria were grown on nutrition broth agar plates at 40ºC for 24 h. The samples before and after solid-state saccharification were collected. The sample was ground with a sterilized pestle and mortar for homogenization, then 0.5 g of sample was mixed with 1 ml sterilized deionized water. The suspension was then serially diluted and spread on to the plates with five replications.

3-2-8. DNA extraction

Xiaoqu and samples during solid-state saccharification (corresponding to 2.5 g of raw rice) were used. The saccharification sample was taken at 0, 8, 16, and 24 h. The taken sample was frozen with liquid nitrogen then ground in a sterilized pestle and mortar. The total DNA of the 0.4 g ground sample was extracted in five replications using a FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). The DNA of the 0.4 g *xiaoqu* powder was also extracted in triplicate with the same kit. The DNA concentration was measured using a NanoDrop 8000 UV Visible Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

3-2-9. Selective PCR amplification of fungal and bacterial DNA

Selective amplification of fungal and bacterial DNA was carried out by applying locked nucleic acid (LNA) mediated PCR developed by Ikenaga *et al*. (Ikenaga and Sakai, 2014; Ikenaga *et al*., 2015, 2016a and 2018). This technique enables to suppress the DNA amplification derived from rice. The PCR mixtures for fungi contained the extracted DNA, premix Ex Taq Hot Start Version (Takara Bio, Shiga, Japan), ITS1F KU LNA and ITS4 primers (0.8 μM as final concentration each), ITS4 LNA oligonucleotides (4.0 μM as final concentration), and sterilized ultrapure water (Ikenaga *et al*., 2016a). The PCR program consisted of initial denaturation at 94ºC for 3 min, followed by 40 cycles of denaturation at 94ºC for 30 s, annealing of LNA oligonucleotides at 70ºC for 30 s, annealing of primers at 54ºC for 30 s, with a final extension at 72ºC for 1.5 min. The PCR mixtures for bacteria contained the extracted DNA, premix Ex Taq Hot Start Version (Takara Bio), DNA primers KU68f and KU1494r (0.8 μM as final concentration each), LNA oligonucleotides Mit63a, Mit1492a, Pla63a, and Pla1492a (4.0 μM as final concentration each), and sterilized ultrapure water (Ikenaga *et al*., 2016b and 2018). PCR program was also composed of denaturation, annealing of LNA oligonucleotides, annealing of DNA primers and extension. The LNA-PCR products were purified by NucleoSpin Gel and PCR Clean-

up (Macherey-Nagel, Düren, Germany) after being confirmed through electrophoresis on a 1.5% agarose gel. All primers and LNA oligonucleotides sequences are shown in Table 3-1.

3-2-10. Denaturing gradient gel electrophoresis

The purified LNA-PCR products were applied for the nested PCRs. The primers set for fungi was ITS1F KU with GC clamp and ITS2 (Ikenaga *et al*., 2016a), and for bacteria 341f-GC and 805r (Muyzer *et al*., 1993; Thijs *et al*., 2017). Approximately 400 ng DNA of the nested PCR product was loaded onto the DGGE system in a DCode universal mutation detection system (BioRad Laboratories, Hercules CA, USA). The gradient of denaturant for fungi was from 15% to 57.5%, and for bacteria from 32.5% to 65%. (100% of denaturant was 7 M urea and 40% (v/v) formamide in 8% (w/v) acrylamide gel) (Muyzer *et al*., 1993). After 14 h of electrophoresis at 100 V and 60ºC, the stained gel by SYBR Gold (Life Technologies Japan, Tokyo, Japan) was imaged under ultraviolet illumination.

3-2-11. Next-generation sequencing

The next-generation sequencing (NGS) analysis for bacteria was performed using a MiSeq system (Illumina, San Diego, CA, USA) with a paired-end method. Amplicon sequencing was performed by Bioengineering Lab. Co., Ltd. (Kanagawa, Japan). The primers set included 341f and 805r (Klindworth *et al*., 2013). The bacterial community analysis of the DNA sequencing data generated on the Illumina was performed using the Qiime pipeline (Caporaso *et al*., 2010). The operational taxonomic units (OTUs) at 97% similarity were clustered at the taxonomic assignment to species. The OTUs proportions in the total sequence number were counted, and less than 1% of the total was compiled as other bacteria. These sequence data were registered as DDBJ DRA number DRA008454, DRA008455, DRA008456, DRA008457, and DRA008458.

3-2-12. Solid-state saccharification using fungal isolates

Isolated fungi from *xiaoqu* were pre-cultured onto a PDA plate at 30ºC for 2 days. The suspension of fungal spores was collected with sterile deionized water. The steamed rice (corresponding to 5 g of raw rice) was prepared in a 200-ml Erlenmeyer flask by autoclaving. One milliliter of spore suspension (3×10^7 cells/ml) was

inoculated into the steamed rice and incubated for 24 h at 35ºC.

3-3. Results and Discussion

3-3-1. Effect of solid-state saccharification on alcohol fermentation

In the preparation of rice-flavor *baijiu*, alcohol fermentation is carried out by adding water and yeast following solid-state saccharification process. To investigate the effects of solid-state saccharification on alcohol fermentation, we compared alcohol fermentation with two prepared mashes. One mash was prepared according to the general process such that alcohol fermentation was carried out following solid-state saccharification. Another mash was prepared by mixing steamed rice, *xiaoqu*, and water simultaneously without solid-state saccharification. We added the pre-cultured yeast used for brewing to both mashes to initiate alcohol fermentation. Alcohol fermentation of the mash prepared using the general process rapidly occurred from the first day and reached a plateau at the 4th day (Fig. 3-1). Coversely, alcohol fermentation in the mash prepared without solid-state saccharification gradually progressed throughout the fermentation period, i.e., it required 11 days to reach the same level as that of the mash prepared by general process. The results of the present study confirm that solid-state

saccharification facilitates alcohol fermentation.

3-3-2. Chemical and biological changes by solid-state saccharification

To assess the chemical and biological changes that occurred following the solid-state saccharification process, we analyzed biochemical composition, enzyme activities, and microbe numbers in the samples before (incubation time of 0 h after mixing steamed rice and *xiaoqu*) and after solid-state saccharification (incubation for 24 h) (Table 3-2). More than 70% of the starch in the rice decomposed into glucose after saccharification. *Rhizopus* sp. was the major fungal species contained in the *xiaoqu* used in the present study. *Rhizopus* sp. has the capacity to saccharify raw native staches because it produces extracellular isoamylase (Ghosh and Ray, 2010). In addition, the extracellular isoamylase can maintain activity under acidic conditions (pH 4). Therefore, *Rhisopus* sp. enhances saccharification rates through the action of isoamylase. In rice-flavor *baijiu* factories, the end of the solid-state saccharification process is determined on the basis of conversion rate of starch to glucose. Generally, when the saccharification rate reaches 70%–85%, the next stage of fermentation is performed (Zheng, 2003). Our results with regard to saccharification rates were consistent with the saccharification rates reported in rice-flavor *baijiu* factories (Hou and Xiao, 1999; Zheng, 2003).

Therefore, our experiments could replicate the conditions in rice-flavor *baijiu* factories.

The numbers of fungal cells increased almost 50-fold after saccharification (Table 3-2). In contrast, bacterial cells were not detected before saccharification, and their numbers barely increased after saccharification. Almost all the fungal colonies detected from *xiaoqu* and the samples after solid-state saccharification were mold, and yeast colonies were hardly observed (data not shown). Therefore, the results indicated that the difference in alcohol fermentation in previous experiments did not arise from differences in yeast cell numbers but from differences in glucose concentrations at the time of initiating fermentation. The low rate of alcohol fermentation increases the risk of contamination by various microbes. The results of the present study demonstrated that solid-state saccharification could prevent such contamination.

Enzyme activities were also compared among the samples before and after the saccharification process. Enzyme activities in the samples before saccharification were too low to measure because the enzymes originated only from *xiaoqu* and were attenuated by the addition of large amounts of steamed rice. To compare the changes in enzyme activity before and after incubation, we estimated enzyme activity in the samples before saccharification from those of *xiaoqu*. The protease, α-amylase, and saccharification activities increased after saccharification compared with those before saccharification, and were 5-fold, 22-fold, and 53-fold higher, respectively. The

enzymes seem to be produced by proliferating fungi. Organic acid concentrations increased following saccharification, with a dramatic increase observed only in lactic acid concentrations.

3-3-3. Changes in microbe compositions during solid-state saccharification

Fungal population proliferation and lactic acid production after saccharification were observed. Lactic acid is generally produced by lactic acid bacteria (LAB). On the other hand, *Rhizopus* sp. is also known as a good lactic acid producer (Vodnar *et al*., 2013). To reveal the lactic acid-producing microbes, the fungal and bacterial compositions in the samples during saccharification were investigated using DGGE and NGS analyses, respectively.

The sample DNA extracts were expected to contain high amounts of DNA derived from rice since rice was the major material in the solid-state saccharification process. PCR by fungal-specific primers and bacterial-specific primers could amplify the rice genomic DNA and the small subunit rRNA genes of rice organelles (mitochondria and plastid), respectively. Therefore, rice DNA in the samples interfered with the amplification of microbial DNA. To eliminate the interference, PCR was performed with the locked nucleic acid technique developed by Ikenaga *et al*. (Ikenaga and Sakai,

2014; Ikenaga *et al*., 2015, 2016a and 2018). The technique resulted in the non-amplification of rice DNA (Figs. 3-2 and 3-3A).

In the DGGE analysis of fungi, *Rhizopus oryzae* isolated from *xiaoqu* was used as a control. The dominant bands corresponded with *R. oryzae* in all the samples (Fig. 3-2). The DGGE patterns during solid-state saccharification were similar to those of *xiaoqu* except at 0 h of saccharification. In the DGGE patterns at 0 h, several different bands were observed. The different bands potentially originated from fungi adhering to rice. In the present study, the rice was heat sterilized by steaming; therefore, no growth of fungi adhering to rice could be observed during the solid-state saccharification. Considering the increase in fungal cell numbers after saccharification, we concluded that *R. oryzae* was preferentially proliferated during saccharification (Table 3-2 and Fig. 3-2).

LAB are potentially responsible for the high amount of lactic acid in the sample after saccharification. Therefore, we investigated bacterial composition during saccharification using DGGE and NGS (Fig. 3-3). Bacterial composition exhibited minor shifts during saccharification; however, the major components were almost similar to those of *xiaoqu* based on either the DGGE pattern or relative abundance following NGS analysis. LAB were barely detected throughout the saccharification process (Fig. 3-3B). *Propionibacterium* sp., *Pseudomonas* sp., and *Ralstonia* sp. were

the major bacteria throughout the saccharification process. The genera have not been reported to produce lactic acid at high quantities (Patrick and McDowell, 2012; Palleroni, 2005; Yabuuchi *et al*., 2005). In addition, during saccharification, *Paenibacillus* sp. and *Bacillaceae* exhibited abrupt increases in their proportions at 8 and 16 h, respectively, while *Staphylococcus* sp. exhibited minor increases in proportions in both the early and late stages. Genus *Paenibacillus* has no capacity to produce lactic acid (Priest, 2009). In the family *Bacillaceae*, only genus *Paraliobacillus* and *Saccharococcus* can produce lactic acid (Logan and Vos, 2009). The genus *Staphylococcus* may produce lactic acid from glucose under anaerobic conditions (Schleifer and Bell, 2009). Although a family *Bacillaceae* and genus *Paraliobacillus* and *Saccharococcus* containing lactic acid-producing bacteria were detected, the proportion was not high. Moreover, bacterial cell numbers after saccharification were few compared to fungal cell numbers (Table 3-2). Therefore, the results suggested that the high amounts of lactic acid present in the samples following saccharification were produced by proliferating *R. oryzae*.

3-3-4. Lactic acid production by the *Rhizopus oryzae* strain isolated from *xiaoqu*

We investigated whether an *R. oryzae* strain isolated from *xiaoqu* could produce

lactic acid. Steamed rice was inoculated with spores of *R. oryzae* in a sterile environment and incubated at 35ºC for 24 h. After incubation, the α-ketoglutaric acid, citric acid, pyruvic acid, malic acid, succinic acid, fumaric acid, and iso-butyric acid concentrations increased slightly while the lactic acid concentrations increased 204-fold (Table 3-3). The tendency to primarily producing lactic acid was similar to the observation in the solid-state saccharification sample.

The results indicated that the lactic acid in the solid-state saccharification sample was produced primarily by *R. oryzae* in *xiaoqu*. Although *Rhizopus* sp. have attracted extensive attention in the production of lactic acid (Abe at el., 2003; Marták *et al*., 2003; Li *et al*., 2005), to the best of our knowledge, the present study is the first to report that *Rhizopus* sp. could also produce lactic acid at high quantities with potential applications in the production of rice-flavor *baijiu*.

3-3-5. Effect of temperature on solid-state saccharification

During the manufacture of rice-flavor *baijiu,* the temperature of the saccharification process is usually maintained at approximately 35ºC in many factories based on experience (Wang, 2003c; Hou and Xiao, 1999; Zheng, 2003). However, the reason for the selection of the temperature has not been explored in research. Therefore, we

investigated the effect of temperature during saccharification to determine the optimal saccharification temperature.

Solid-state saccharification was carried out at different temperatures ranging from 25ºC to 40ºC. After saccharification, reducing sugar and glucose levels in the saccharification samples were the highest at 35ºC (Fig. 3-4A). More than 70% of starch was converted to glucose and more than 80% of starch was converted to reducing sugars. The reducing sugar and glucose levels decreased with increase in temperature, the highest numbers of fungal cells were observed in the saccharification sample at 30ºC, and the numbers decreased with increasing temperature (Fig. 3-4B). Fungal cells hardly grew at 40ºC. Saccharification activity was consistently observed in the saccharification samples maintained at temperatures ranging from 25ºC to 35ºC (Fig. 3-4C). Consequently, glucose levels were greatest at 35ºC due to the higher saccharification activity and higher reaction temperature. Organic acid composition did not vary with temperature and lactic acid was the major organic acid produced (Fig. 3-4D).

Overall, the results demonstrated that solid-state saccharification under the condition favored by factories was appropriate not only for saccharification but also for the production of enzymes and lactic acid, which was accompanied by fungal proliferation. The resultant enzymes are considered to enhance the subsequent alcohol fermentation. Therefore, the temperature in solid-state saccharification systems should

be maintained below 40ºC.

The Japanese liquor manufacturing process begins with the preparation of rice *koji* from *koji* mold (*Aspergillus* sp.). The mold proliferates and produces numerous enzymes required in the process of fermentation. The Japanese liquor *shochu* utilizes black *koji* mold (*Aspergillus luchuensis*) and white *koji* mold (*Aspergillus luchuensis* mut. *kawachii*) in the preparation of rice *koji.* The molds have a high capacity to produce citric acid. Therefore, rice *koji* contains high amounts of citric acid. Citric acid lowers the pH of fermenting mash and could prevent contamination by various bacteria. In addition, it has been reported previously that the low pH of the fermenting mash contributes to the flavor of *shochu* by influencing yeast metabolites present during fermentation and the chemical reactions during distillation (Takamine *et al*., 2018). Therefore, the lactic acid produced during the solid-state saccharification of rice-flavor *baijiu* could have an effect similar to citric acid in *shochu* preparation. The solid-state saccharification process also appears to be similar to the method used for the preparation of rice *koji* (Takamine, 2015).

In the present study, we demonstrated the role of the solid-state saccharification process in the production rice-flavor *baijiu*. The results could facilitate the efficient and stable manufacture of rice-flavor *baijiu*.

3-4. Summary

Rice-flavor *baijiu* is a traditional Chinese liquor that is manufactured using a solid-state saccharification process. In the present study, we investigated the role of the process in making of rice-flavor *baijiu* using chemical and biological quantitative analysis. More than 70% of starch in rice decomposed to glucose after saccharification. In addition, the number of fungal cells, saccharification activity, and lactic acid concentrations increased. *Rhizopus oryzae* was identified as the major fungus proliferating under saccharification based on denaturing gradient gel electrophoresis analysis targeting the internal spacer transcribed region. Lactic acid bacteria were not detected by 16S rRNA gene-based next-generation sequencing analysis during saccharification. Conversely, *R. oryzae*, isolated from *xiaoqu,* exhibited a capacity to produce lactic acid. The results imply that the solid-state saccharification is essential not only for saccharification but also for the culture of *R. oryzae*, which promotes saccharification activity and lactic acid production. We also investigated the most appropriate temperature for solid-state saccharification and the optimum temperature for *R. oryzae* cultivation, enzyme production, and saccharification was 35ºC. The results could facilitate the efficient and stable manufacture of rice-flavor *baijiu*.

TABLE 3-1. Primers and LNA oligonucleotides used in the investigation of microbial communities.

			Sequences (5‘-3‘)	Ref
Bacterial SSU rRNA genes	LNA-PCR	DNA Primer KU68f	AYACATGCAAGTCGARCG	1
		DNA Primer KU1494r	GTCGTAACAAGGTARCC	
		LNA oligonucleotides Mit63a	GTCGA**A**CG**T**TGT**TT**T**C**G**G**p	2
		LNA oligonucleotides Mit1492a	CTTC**A**CCCCA**G**TCG**AA**G**A**p	
		LNA oligonucleotides Pla63a	GTCG**A**ACGG**G**AAG**T**GG**T**p	
		LNA oligonucleotides Pla1492a	CTTCAC**T**CCA**G**TCGC**A**AG**C**p	
	Nested PCR for DGGE	DNA Primer 341f-GC	CGCCCGCCGCGCGCGGCGGGCGG GGCGGGGGCACGGGGGGCCTACG GGAGGCAGCAG	3
		DNA Primer 805r	GACTACHVGGGTATCTAATCC	4
	NGS	DNA Primer 341f	ACACTCTTTCCCTACACGACGCTC TTCCGATCT-NNNNN-CCTACGGGNGGCWGCAG	5
		DNA Primer 805r	GTGACTGGAGTTCAGACGTGTGC TCTTCCGATCT-NNNNN-GACTACHVGGGTATCTAATCC	
Fungal ITS region genes	LNA-PCR	LNA primer ITS1F KU	CTYG**G**TCATTTAGAGGAAS**T**A**A**	6
		DNA Primer ITS4	TCCTCCGCTTATTGATATGC	
		ITS4 LNA oligonucleotides a	CTTAA**AC**TCAGCGGGTAGTCC**C**p	
	Nested PCR for DGGE	DNA Primer ITS1F KU with GC clamp	CGCCCGCCGCGCGCGGCGGGCGG GGCGGGGGCACGGGGGGCTYGG TCATTTAGAGGAASTAA	
		DNA Primer ITS2	GCTGCGTTCTTCATCGATGC	
	NGS	DNA Primer gITS7	ACACTCTTTCCCTACACGACGCTC TTCCGATCT-NNNNN-GTGAATCATCGARTCTTTG	7
		DNA Primer ITS4	GTGACTGGAGTTCAGACGTGTGC TCTTCCGATCT-NNNNN-TCCTCCGCTTATTGATATGC	

Bold font in the sequence of LNA oligonucleotides and LNA primer indicated the LNA base instead of the DNA base. The small letter p in the 3' end of all LNA oligonucleotides indicated the phosphorylation to avoid the extension during PCR. Ref: 1. Ikenaga *et al*., 2018; 2. Ikenaga *et al*., 2016b; 3. Muyzer *et al*., 1993; 4. Thijs *et al*., 2017; 5. Klindworth *et al*., 2013; 6. Ikenaga *et al*., 2016a; 7. Bokulich *et al*., 2013.

TABLE 3-2. Enzyme activities, compound levels, and numbers of microbial cells before and after solid-state saccharification.

			Solid-state saccharification	
		Xiaoqu	Before (incubation of 0 h)	After (incubation of 24 h)
Glucose (mg/g)		-	1.71 ± 0.09	220.90 ± 22.01
Microbial cells (CFU/g)	Fungal cells	-	94 ± 12	4365 ± 1478
	Bacterial cells	-	ND	56 ± 23
Enzymes activity (Unit/g)	Protease	6574.27 ± 207.02	(31.61 ± 1.00)	152.03 ± 19.95
	α-Amylase	88.90 ± 3.40	(0.42 ± 0.02)	9.14 ± 1.05
	Saccharification activity	87.24 ± 2.81	(0.42 ± 0.01)	22.42 ± 0.67
Organic acids (mg/g)	α-Ketoglutaric acid	-	0.068 ± 0.008	0.131 ± 0.004
	Citric acid	-	ND	0.099 ± 0.010
	Malic acid	-	0.071 ± 0.005	0.242 ± 0.030
	Succinic acid	-	ND	0.233 ± 0.009
	Fumaric acid	-	ND	0.495 ± 0.017
	Lactic acid	-	ND	4.866 ± 0.227

Data are means ± SD. Microbial enumeration was performed on five plates. The other determinations were performed in triplicate. The enzyme activities of the samples before solid-state saccharification were calculated from those of *xiaoqu* and are represented in parentheses. ND means not detected.

TABLE 3-3. Organic acids contents before and after solid-state saccharification using *Rhizopus oryzae* isolated from *xiaoqu*.

Organic acid (mg/g)	Before	After
α-Ketoglutaric acid	0.053 ± 0.004	0.055 ± 0.002
Citric acid	0.029 ± 0.004	0.041 ± 0.008
Pyruvic acid	ND	0.043 ± 0.022
Malic acid	0.030 ± 0.006	0.061 ± 0.009
Succinic acid	ND	0.069 ± 0.007
Fumaric acid	ND	0.095 ± 0.016
iso-Butyric acid	ND	0.118 ± 0.020
Lactic acid	0.055 ± 0.013	11.223 ± 0.289

Data are means ± SD. Determination was performed in triplicate.

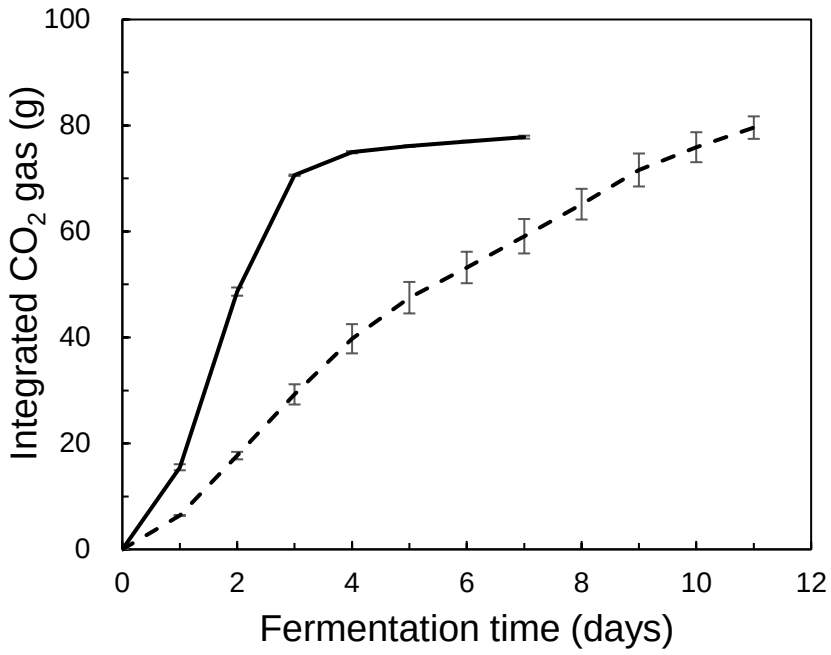

FIG. 3-1. Time course of alcohol fermentation. Alcohol fermentation of rice-flavor *baijiu* was carried out either before (dotted line) or after (solid line) solid-state saccharification. Alcohol fermentation was monitored by determining the amount of carbon dioxide gas generated, which was calculated by measuring the reduction in weight of the fermented sample every day. The integration curve for the weight reduction was plotted on a graph. Vertical bars indicate the standard deviations of three independent experiments.

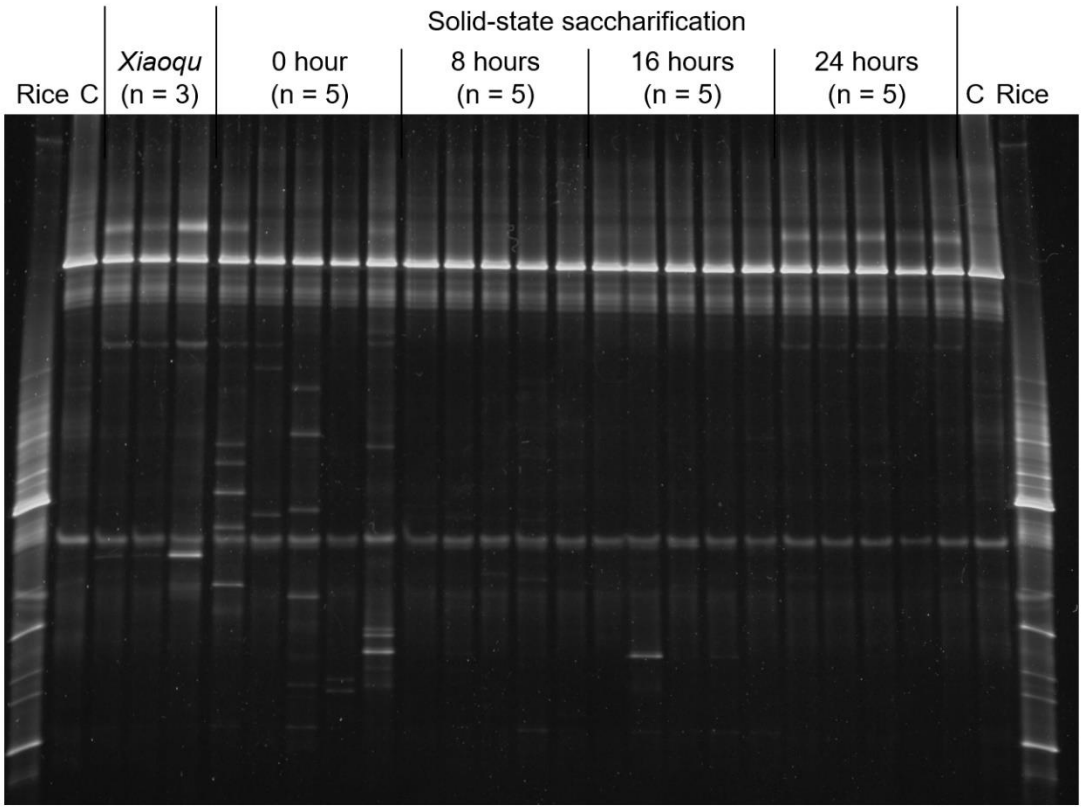

FIG. 3-2. Denaturing gradient gel electrophoresis (DGGE) pattern of fungal ITS1 regions in *xiaoqu* (n = 3) and samples that underwent solid-state saccharification for 0, 8, 16, and 24 h (n = 5). C represents the ITS1 genes of *Rhizopus oryzae* isolated from *xiaoqu*. Rice represents amplicons derived from aseptic rice.

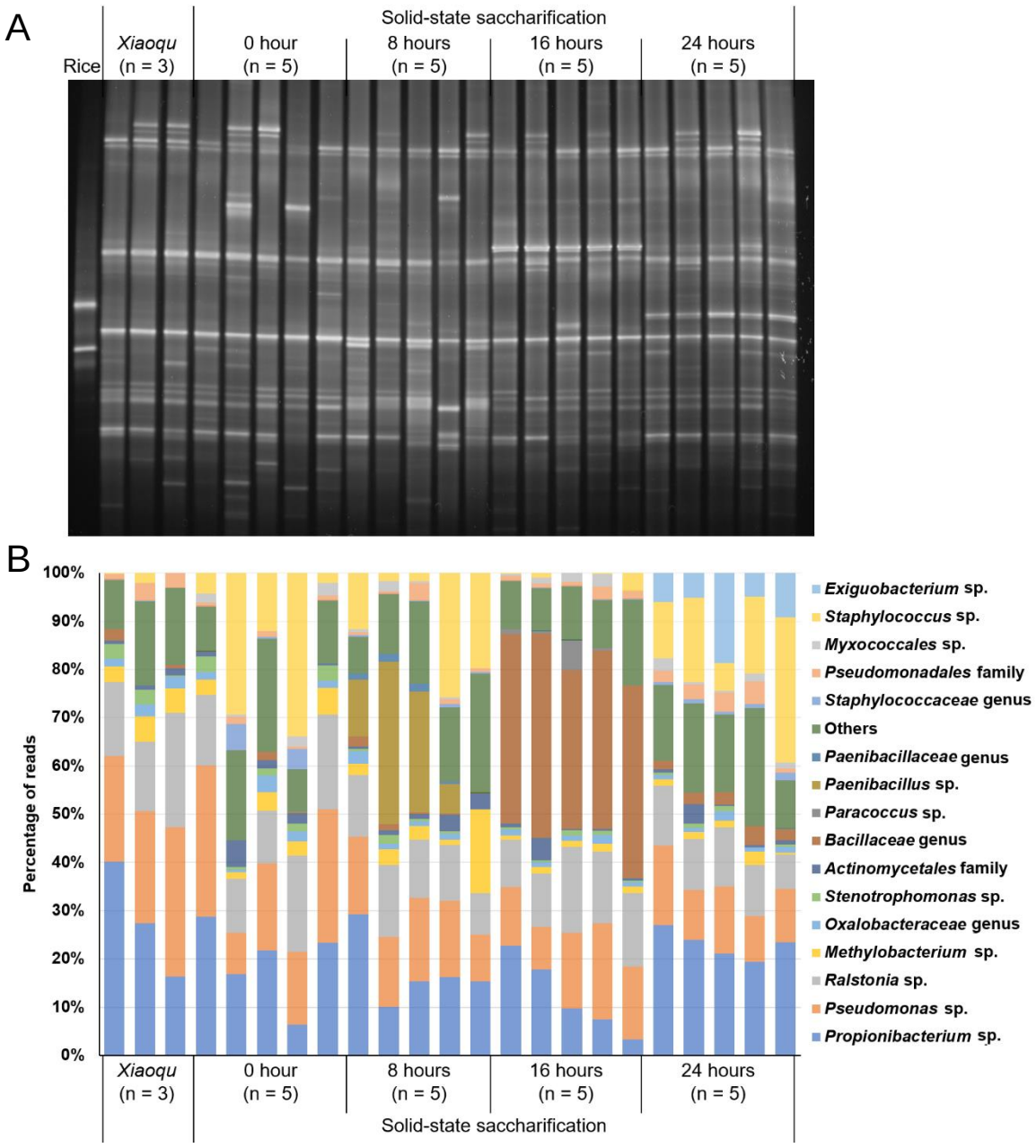

FIG. 3-3. Bacterial composition in *xiaoqu* (n = 3) and samples that underwent solid-state saccharification for 0, 8, 16, and 24 h (n = 5). A, the denaturing gradient gel electrophoresis (DGGE) pattern of bacterial SSU rRNA genes in *xiaoqu* and samples that underwent solid-state saccharification for 0, 8, 16, and 24 h. Rice means aseptic rice organelle SSU rRNA genes. B, the percentages of reads (order, family, and genus levels) for the bacterial genes in *xiaoqu* and samples that underwent solid-state saccharification for 0, 8, 16, and 24 h were determined by next-generation sequencing (NGS) of SSU rRNA genes.

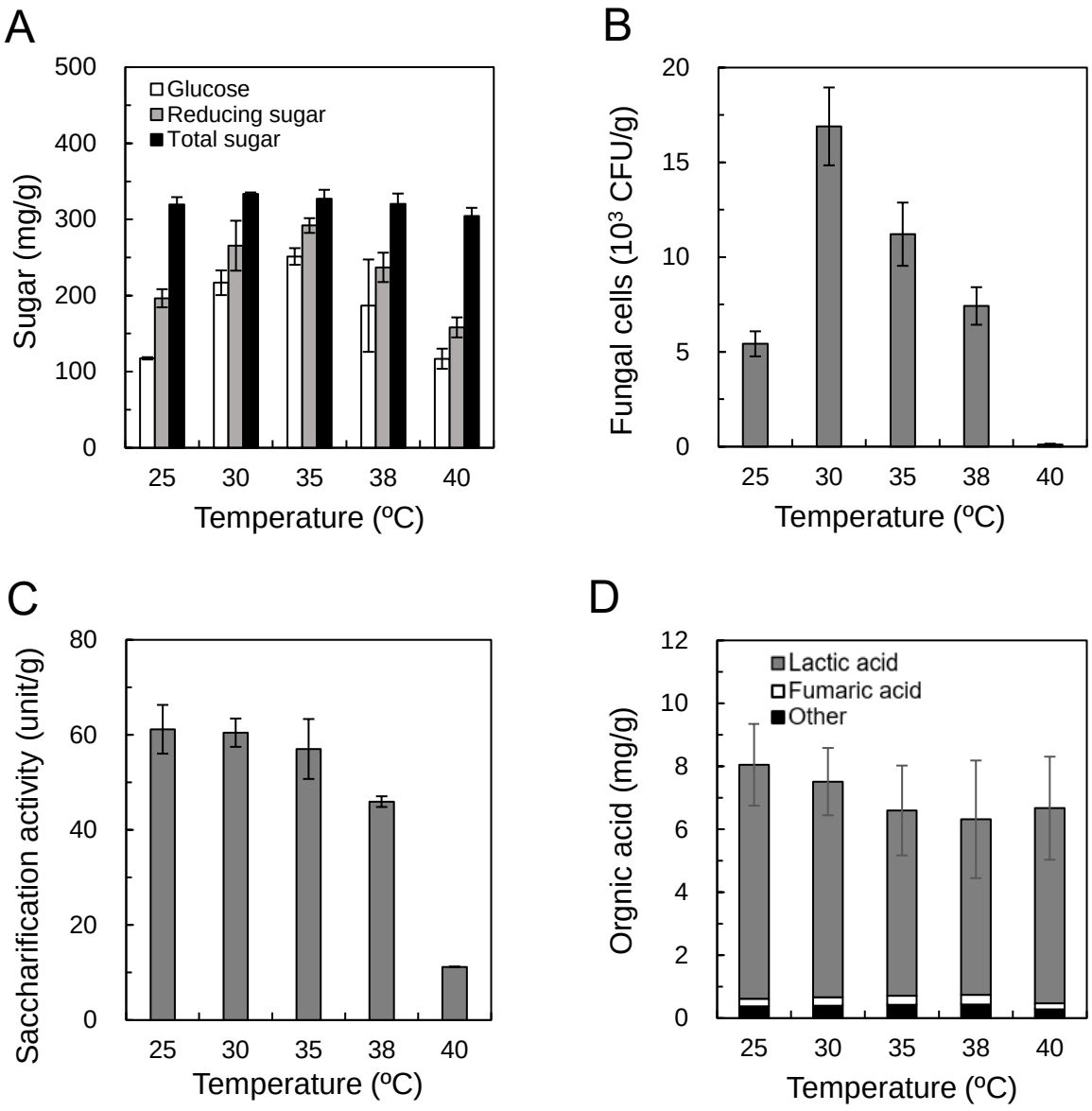

FIG. 3-4. Chemical and biological analysis of samples after solid-state saccharification at different temperatures. A, glucose (open bars), reducing sugar (shaded bars), and total sugar (closed bars) concentrations were measured by three independent experiments. B, the number of fungal cells was measured by culturing them on agar plate media (n=5). C, saccharification activity was measured by three independent experiments. D, the fumaric acid (open bars), lactic acid (shaded bars), and other organic acid (closed bars) concentrations were determined based on ten independent experiments. Vertical bars indicate the standard deviations. The standard deviations shown in panel D are for lactic acid.

Chapter 4. Discussion

In this study, we revealed the chemical and flavor profiles of rice-flavor *bajiu* by analyzing the components of commercial liquor in chapter 2. Moreover, we also investigated the role of the unique solid-state saccharification process in making of rice-flavor *baijiu* in chapter 3.

Compared to *awamori* and *kome-shochu*, the most characteristic components in rice-flavor *baijiu* were acetic acid, lactic acid, and ethyl lactate. The concentration of acetic acid in rice-flavor *baijiu* was 6 times higher than the average concentration of three other types: sauce-, strong-, and light-flavor *baijiu* (Fang *et al*., 2019). These results were indicated that acetic acid is one of the important compounds in rice-flavor *baijiu*. Unlike other three types *baijiu* and Japanese liquors, which are saccharified and fermented simultaneously, rice-flavor *baijiu* is solid-state saccharification prior to liquid-state fermentation. The glucose content in the fermented mash of rice-flavor *baijiu* is calculated 14% (w/v) at the beginning of the fermentation from our research. Therefore, it is expected that the yeast receives a higher sugar stress than other three types *baijiu* and Japanese liquors at the beginning of the fermentation. It is previously reported that the high sugar stress is up-regulated the gene expression of acetic acid from ac*et al*dehyde (Erasmus *et al*., 2003). The acetate formation plays a role in

maintaining the redox balance in yeast cells (Van and Scheffers, 1986). Thereby, it is implied that the high glucose stress induces the high amount of acetic acid in rice-flavor *baijiu*.

Lactic acid was detected in all rice-flavor *baijiu* samples with an average value of 666 mg/L. Equivalently, lactic acid was the major non-volatile acid in other three types with 125~484 mg/L (Fang *et al*., 2019). These concentrations are too high to explain by an entrainment from the mash during distillation. Therefore, we speculated that the artificial addition of lactic acid was a uniform means to adjust the flavor of *baijiu*.

Ethyl lactate was not only an important component of rice-flavor *baijiu* to distinguish *awamori* and *kome-shochu* but a common characteristic component of *baijiu*. It is expected that the ethyl lactate in *baijiu* is commonly produced from lactic acid produced by *Rhizopus* sp. or lactic-acid bacteria during fermentation. There were 379 mg/L in rice-flavor *baijiu*, 916 mg/L in sauce-flavor *baijiu* (Fang *et al*., 2019), 778 mg/L in strong-flavor *baijiu* (An *et al*., 2019), and 1081 mg/L in light-flavor *baijiu* (An *et al*., 2019). Although ethyl lactate level of rice-flavor *baijiu* was a little low among other tapes of *baijiu*, it is totally one of the large amount compounds in rice-flavor *baijiu*. Rice-flavor *baijiu* generally contains smaller number and lower level of volatile compounds than other type of *baijiu* (Wu, 2001; Shao *et al*., 2005; Liu and Sun, 2018; An *et al*, 2019). It means that ethyl lactate has a larger impact of flavor in rice-flavor

baijiu than in other type of *baijiu*. These show that ethyl lactate must be a key compound in rice-flavor *baijiu.* Our results suggest that this ethyl lactate level in rice-flavor *baijiu* related to the solid-state saccharification of the production process.

We could show that the property of manufacturing process in rice-flavor *baijiu* affect to the characterization of flavor. In the future, it will be necessary to identify the formation mechanisms of acetic acid and ethyl lactate during the making of rice-flavor *baijiu*.

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謝辞

本研究の遂行および本論文をまとめにあたり，懇切なる御指導･御鞭撻を賜りました鹿児島大学 農学部 附属焼酎・発酵学教育研究センター 焼酎製造学部門 髙峯和則 教授に深く感謝の意を表します。また，本論文の審査および御指導賜りました同研究部門 吉﨑由美子 准教授および佐賀大学 農学部 北垣浩志 教授に心より厚く御礼申し上げます。本論文の審査および御校閲を賜りました琉球大学 農学部 外山博英 教授および鹿児島大学 農学部 玉置尚徳 教授に厚く感謝致します。さらに，本研究に関して有益な御助言を賜りました鹿児島大学 農学部 附属焼酎･発酵学教育研究センター 焼酎製造学部門 奥津果優 特任助教と，直接研究に御協力頂きた藏薗秀伍 氏，杉町美奈 氏および竹内春佳 氏をはじめとするに焼酎学研究室の学生諸氏に心から御礼申し上げます。

また，本研究遂行にあたり，LNA オリゴによる PCR クランプ技術および次世代シーケンス解析の方法を御教授頂きました鹿児島大学 農学部 池永誠 准教授に厚く御礼申し上げます。本研究の共同研究者の中国食品発酵工業研究院 韓興林 白酒研究員にも感謝の意を表します。

さらに， 留学生での博士課程の機会を頂くとともに， 多大なる御支援を頂きました両親に深謝致します。

最後に， 本研究の御縁と賜り， 研究に対する御指導， 論文の作成に御教示および生活に御支援を頂きました鹿児島大学 農学部 附属焼酎･発酵学教育研究センター 焼酎製造学部門 吉﨑由美子 准教授に再度感謝申し上げます。