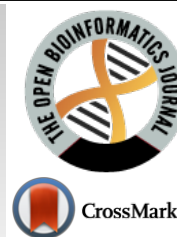




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## RESEARCH ARTICLE

### Bacterial DNA Diversity among Clear and Cloudy Sakes, and *Sake-kasu*

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#### Abstract:

#### Background:

The traditional Japanese alcoholic drink, sake, is classified into two types: those that contain sediment produced during the production process (cloudy sakes) and those that do not contain such sediment (clear sakes). Leftover pressed sediment from the sake production process, *sake-kasu* (sake cake or sake lees), is commercially available and is highly nutritious for humans.

#### Objective:

The purpose of this study was to determine the difference among component bacterial DNA sequences of clear and cloudy sakes, and *sake-kasu*.

#### Methods:

We compared the 16S rDNA sequences from 44 samples of clear sake, 3 samples of cloudy sake, and 11 samples of *sake-kasu*.

#### Results:

The DNA sequences were divided into three major clusters; however, sequences in *sake-kasu* were located in just one cluster forming two lineages. The microbial diversity in *sake-kasu* was lower than that in clear and cloudy sakes, which may be because some of the contaminating bacterial cells do not lyse during the production process and remain intact, along with yeast cells, in *sake-kasu*.

#### Conclusion:

Bacterial DNA frequently detected in sake samples was from environmental bacterial contamination that occurs early in the sake production process. Contaminating bacteria are usually killed by the ethanol produced as the sake yeast grows; after which, if bacteria lyse, the bacterial DNA is released into the sake solution. However, if the bacterial cells do not lyse, they will precipitate toward the sediment. Thus, there is bacterial DNA diversity in clear and cloudy sake, but less diversity in *sake-kasu*.

**Keywords:** Bacterial contamination, Sake production process, Bacterial cell lysis, *Sake-kasu*, 16S rDNA, Japanese alcoholic drink.

#### Article History

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## 1. INTRODUCTION

*Koji*, *moto*, rice, and water are needed for sake production and no bacteria are added. However, a diverse range of bacterial DNA sequences were detected in sake samples previously [1 - 6]. This indicates that bacterial contamination occurs during sake production. The fungus, *Aspergillus oryzae* (*Aspergillus flavus* subsp. *flavus* var. *oryzae*), produces *koji*, which converts rice starch into sugars [7, 8]. The ethanol fermentation starter, *moto*, is produced using *koji* and the sake yeast, *Saccharomyces cerevisiae*, which converts sugars to ethanol [9]. There are no reports about bacterial ethanol

fermentation during sake production.

While all sakes are filtered, clear sake is completely filtered, while cloudy sake is not. Thus, the cloudy sake contains sediments produced during the sake production process. After the filtered sediments are pressed, *sake-kasu* is produced. Clear sake, cloudy sake, and *sake-kasu* are shown in Fig. (1). Many cloudy types of sakes effervesce when opened, indicating fermentation by the yeast occurred. Effervescence is not seen in clear sakes. *Hiire* (pasteurization) is usually performed once before storage and once before bottling for sterilization. On the other hand, cloudy sake is not pasteurized. We examined bacterial DNA in different sake samples during the sake production process [3, 4, 6]. Bacterial contamination mostly occurs during *koji* and *moto* production [6]. At the

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genus level, *Acinetobacter*, *Bacillus*, and *Staphylococcus* DNA were frequently detected during the *koji* production process, while DNA from *Lactobacillus*, *Leuconostoc*, *Pseudomonas*, *Sphingomonas*, *Thiobacillus*, and *Vibrio* bacteria was frequently detected during the *moto* production process [1, 3]. The lactic acid bacterial DNA is detected more frequently in sake, using *yamahai-moto* than *sokujo-moto* [4].

In this study, we compared the bacterial DNA diversities of 11 samples of *sake-kasu*, 44 samples of clear sake, and 3 samples of cloudy sake.

## 2. MATERIALS AND METHODS

### 2.1. Sake Samples

We compared the 16S rDNA sequences from 44 samples of clear sake, 3 samples of cloudy sake, and 11 samples of *sake-kasu* from different 33 breweries in different 13 prefectures (Table 1).

### 2.2. DNA Isolation and Purification

DNA was isolated and purified from clear and cloudy sakes according to the protocol used in previous studies [3, 4]. The isolated DNA was concentrated using Amicon Ultra 15

Centrifugal Filter Units (Merck Millipore, Darmstadt) or Nanosep 10K ultrafiltration unit (PALL, Port Washington). DNA was purified using the NucleoSpin gDNA Clean-up kit (Macherey–Nagel, Duren). Samples of *sake-kasu* were crushed using Multi-beads Shocker (Yasui Kikai, Osaka, Japan) (3,000 rpm, 10 cycles: 60 s ON and 60 s OFF). After the samples were crushed, 60  $\mu$ L of C1 solution (150 mM NaCl, 4% SDS, 0.5 M Tris) was added and the samples were centrifuged at 10,000  $\times$  g for 10 min. Subsequently, 250  $\mu$ L of the supernatant and C2 solution (133 mM ammonium acetate) were mixed. The mixture was incubated at 4°C for 10 min and then centrifuged at 10,000  $\times$  g for 5 min. The supernatant and 200  $\mu$ L of C3 solution (120 mM ammonium aluminum sulfate dodecahydrate) were mixed. The mixture was incubated at 4°C for 10 min, and then centrifuged at 10,000  $\times$  g for 5 min. Thereafter, 800  $\mu$ L of C4 solution (5 M GuHCL, 30 mM Tris, 9% isopropanol) was added per 500  $\mu$ L of the supernatant. The mixture was passed through a FastGene Plasmid Mini Kit mP Column (Nippon Genetics, Tokyo, Japan), followed by the addition of 700  $\mu$ L of solution C5 (10 mM Tris, 100 mM NaCl, 50% EtOH), centrifugation at 10,000  $\times$  g for 2 min, and washing three times. Finally, DNA was eluted using 100  $\mu$ L of TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0).

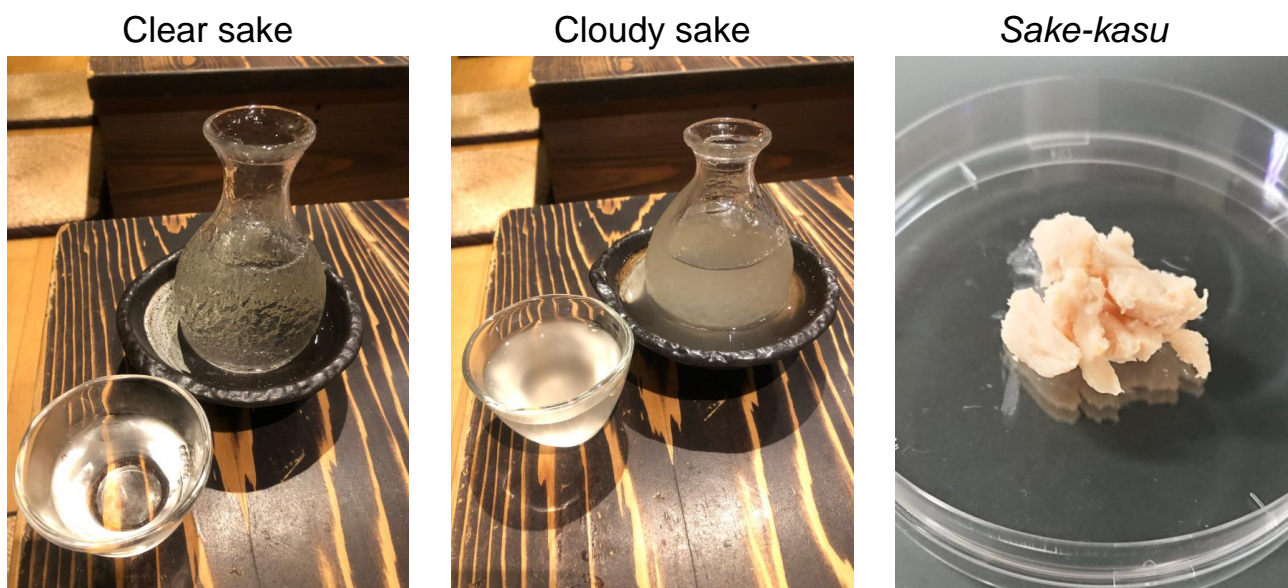


Fig. (1). Photos of clear sake, cloudy sake, and *sake-kasu*.

Table 1. List of samples used in this study.

Sample	Type	Pasturization Times	Brewery	Reference
A01	clear sake	2	Toyama 1	This study
A01+	clear sake	2	Toyama 1	This study
A02	clear sake	0	Toyama 1	This study
A03	clear sake	1	Toyama 1	This study
A04	clear sake	1	Toyama 1	[3]
A05	clear sake	2	Toyama 1	This study
A06	clear sake	2	Toyama 1	[3]

(Table 3) cont.....

Sample	Type	Pasturization Times	Brewery	Reference
A07	clear sake	0	Toyama 1	[3]
A08	clear sake	2	Toyama 1	[3]
A09	clear sake	2	Toyama 1	[3]
A10	cloudy sake	0	Toyama 1	[3]
A11	clear sake	2	Toyama 1	[3]
B01	clear sake	2	Toyama 2	[3]
C01	clear sake	0	Toyama 3	[3, 4]
D01	clear sake	2	Aichi 1	[3]
E01	clear sake	2	Iwate 1	[3]
F01	clear sake	2	Akita 1	[3]
F1	sake-kasu	-	Iwate 1	This study
F2	sake-kasu	-	Akita 2	This study
F3	sake-kasu	-	Tottori 1	This study
F4	sake-kasu	-	Toyama 1	This study
F5	sake-kasu	-	Ishikawa 1	This study
F6	sake-kasu	-	Akita 3	This study
F7	sake-kasu	-	Mie 1	This study
F8	sake-kasu	-	Akita 4	This study
F9	sake-kasu	-	Nara 1	This study
F10	sake-kasu	-	Nara 2	This study
F11	sake-kasu	-	Toyama 4	This study
G01	clear sake	2	Gifu 1	[3]
H01	clear sake	unknown	Fukui 1	This study
I01	clear sake	2	Ishikawa 2	[3, 4]
J01	clear sake	2	Iwate 2	[3]
K01	clear sake	2	Iwate 3	[3]
L01	clear sake	2	Iwate 4	[3]
M1	cloudy sake	0	Toyama 1	This study
M2	clear sake	2	Nara 2	This study
M3	clear sake	2	Aomori 1	This study
M4	clear sake	2	Aichi 2	This study
M5	clear sake	2	Kyoto 1	This study
M6	clear sake	2	Kyoto 2	This study
M7	clear sake	2	Aomori 2	This study
M8	clear sake	2	Aichi 3	This study
M9	clear sake	0	Iwate 5	This study
M10	clear sake	2	Ishikawa 5	This study
M11	clear sake	2	Kyoto 3	This study
M12	clear sake	2	Ishikawa 3	This study
M13	clear sake	2	Nara 3	This study
M14	clear sake	0	Yamanashi 1	This study
M15	clear sake	2	Yamanashi 1	This study
M16	clear sake	2	Ishikawa 4	This study
M18	clear sake	2	Toyama 1	This study
M19	clear sake	2	Ishikawa 2	[4]
M20	clear sake	0	Toyama 3	[4]
M21	cloudy sake	0	Toyama 1	This study
M22	clear sake	0	Iwate 1	This study
M23	clear sake	0	Iwate 1	This study
M24	clear sake	2	Iwate 1	This study
M25	clear sake	2	Iwate 1	This study

**Table 2. Bacterial growth media used for bacterial isolation from *Hatsuzoe*.**

Name	Component	Concentration
LB	tryptone	10 g/L
	NaCl	10 g/L
	yeast extract	5 g/L
MB	peptone	5 g/L
	yeast extract	1 g/L
	ferric citrate	0.1 g/L
	NaCl	19.45 g/L
	MgCl <sub>2</sub>	5.9 g/L
	MgSO <sub>4</sub>	3.24 g/L
	CaCl <sub>2</sub>	1.8 g/L
	KCl	0.55 g/L
	NaHCO <sub>3</sub>	0.16 g/L
	KBr	0.08 g/L
	SrCl <sub>2</sub>	0.034 g/L
	H <sub>3</sub> BO <sub>3</sub>	0.022 g/L
	Na <sub>2</sub> HPO <sub>4</sub>	0.008 g/L
	Na <sub>2</sub> SiO <sub>3</sub>	0.004 g/L
	NaF	0.0024 g/L
NH <sub>4</sub> NO <sub>3</sub>	0.0016 g/L	
TGY	tryptone	5 g/L
	yeast extract	3 g/L
	glucose	1 g/L

### 2.3. Bacterial Isolation From *Hatsuzoe*

Bacterial isolation was performed from 6 different *Hatsuzoe* (the first mixture of *koji* and *moto*) samples of Brewery Toyama 1. Three different media (Table 2) containing 25 µg/mL cycloheximide were used for the isolation. Each *Hatsuzoe* sample (30 µL) was added to each medium (20 mL) and cultured at 15 °C and 30 °C for 72 h. After that, a single colony was chosen.

### 2.4. Massively Parallel DNA Sequencing

V3 and V4 regions of the 16S rRNA gene from each DNA fraction were amplified by PCR and DNA sequencing performed according to a previous study [3, 4].

### 2.5. Analysis of Bacterial DNA Sequence Components

Using the protocol developed in a previous study, sequencing data were analyzed using QIIME (Quantitative Insights into Microbial Ecology, version 1.9.0) [10]. The resultant sequence was analyzed by length (from 400 to 500 bp) using the statistical software R (<http://www.R-project.org/>) with the Biostrings package [11]. Next, chimera sequences were removed using QIIME with the Greengenes reference database (<http://greengenes.lbl.gov/>, v13.8), and the “non\_chimeras\_retention” parameter set as “intersection”. OTU-picking and alpha-diversity calculations were then performed against the 16S rRNA genes database (Greengenes database, version 13.8). The resultant sequences were subjected to BLASTn search (version 2.9.0+) and alignment against the 16S Microbial NCBI database as installed on June 9<sup>th</sup>, 2019.

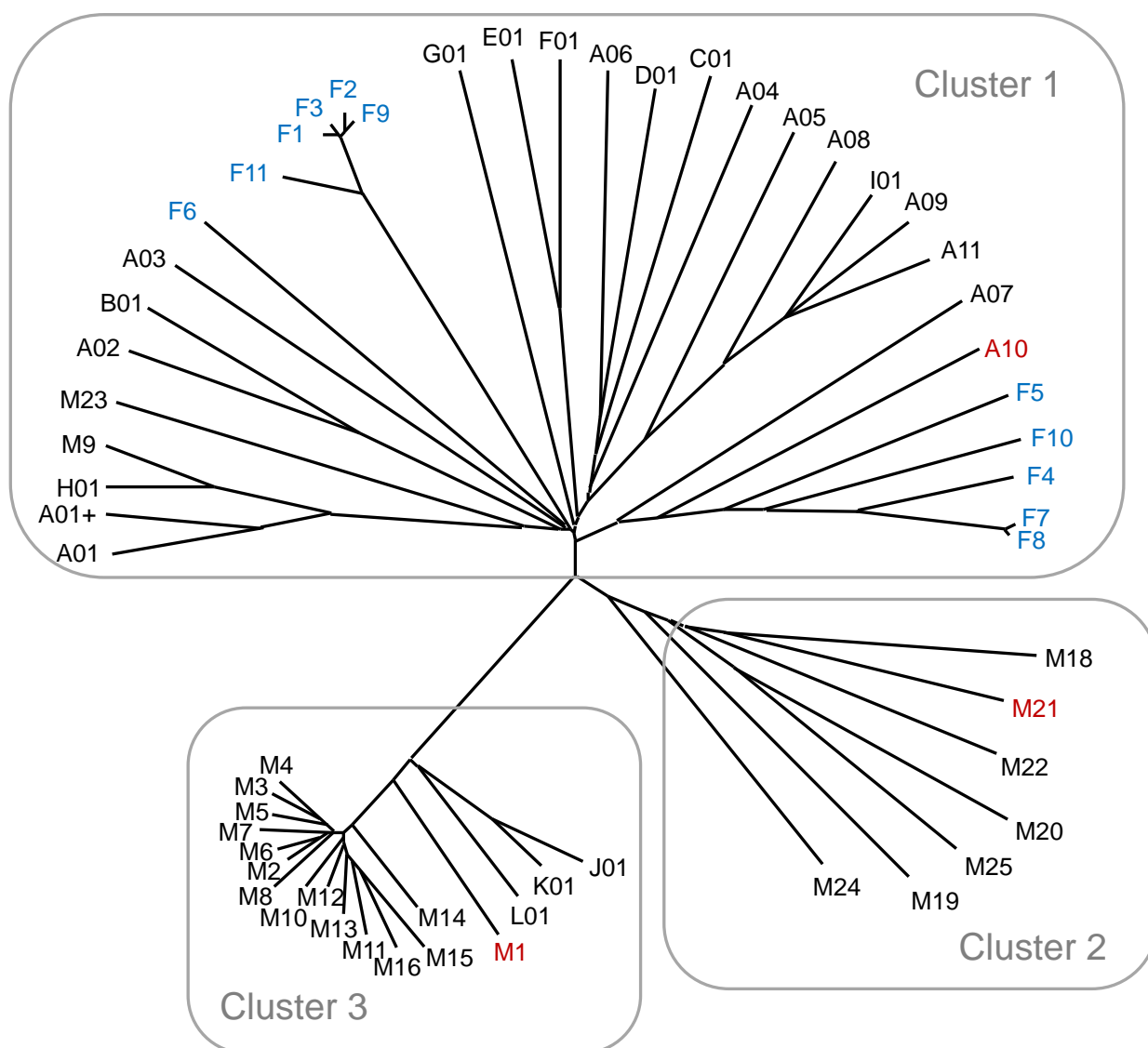
### 2.6. Cluster Analysis

Based on the results from the analysis of the bacterial DNA, sequence frequencies were obtained (Suppl. Table 1). Each frequency vector consisted of 1,611 elements. Each vector was converted into a unit vector. The Euclidean distance between different two vectors (samples) among 58 vectors was calculated using the statistical software R (<http://www.R-project.org/>). On the basis of the distance matrix, a neighbor-joining tree was constructed using the MEGA software [12].

## 3. RESULTS AND DISCUSSION

A total of 1,611 bacterial DNA sequences were obtained in the 58 samples. Based on the result, we rearranged the bacterial DNA sequences at the genus level. The DNA sequences of less than 1% are summarized as others in (Suppl. Table 2). As a result, 168 bacterial genera were identified (Suppl. Table 2).

Bacterial DNA sequence components were grouped into three major clusters (Fig. 2). Cluster 1 is the largest, consisting of 33 samples (22 samples of clear sake, 11 samples of *sake-kasu*, and 1 sample of cloudy sake), while cluster 2 consisted of 6 samples of clear sake and 1 sample of cloudy sake, and cluster 3 of 17 samples of clear sake and 1 sample of cloudy sake. Although the samples of clear and cloudy sakes were distributed into three different clusters, the samples of *sake-kasu* were distributed into just one cluster with two different lineages (Fig. 2). The microbial sequences in *sake-kasu* and cloudy sake were not separate from that of clear sake, indicating that no bacteria are specific to *sake-kasu* or cloudy sake.



**Fig. (2).** Phylogenetic relationships among clear sake, cloudy sake, and *sake-kasu*. This phylogenetic tree was constructed based on the bacterial DNA sequence component in each sample of clear sake, cloudy sake, and *sake-kasu*.

The most frequently detected bacterial genus in this study was *Pseudomonas*, detected in 40 out of the total 58 samples, and in a range of between 1.7% of sample M18 and 40.3% of sample A11 (Suppl. Table 2), (Fig. 3). However, *Pseudomonas* was not detected in any of the 11 *sake-kasu* samples (Suppl. Table 2). *Pseudomonas* has been detected in various environments [13]. *Delftia*, *Loriellopsis*, and *Rhodoligotrophos* were also frequently detected (Suppl. Table 2). Although *Delftia* was detected in 28 samples, and in a range of 1.0% of sample M10 and 14.3% of sample D01, it was not detected in the *sake-kasu* samples (Suppl. Table 2), Fig. (3). However, *Loriellopsis* and *Rhodoligotrophos* were both detected in the *sake-kasu* samples, with detection rates exceeding 20% in the *sake-kasu* samples F4, F5, F7, F8, and F10 (Suppl. Table 2). *Loriellopsis* and *Rhodoligotrophos* were isolated from low-temperature environments (below 20 °C) [14, 15]. Although a wide range of bacteria were detected in sake and *sake-kasu*,

this was from bacteria usually present in the environment.

The genus *Kocuria* was detected in only three samples: A10, F4, and M21 (Suppl. Table 2). Interestingly, these samples were from the same sake brewery (Toyama 1) (Table 1). In addition, the samples A10 and M21 are both cloudy types of sake, while F4 is *sake-kasu* (Table 1). *Kocuria* was isolated from the first mixture of *koji* and *moto* from Brewery Toyama 1 [6]. Thus, this genus may be specific to sake from Brewery Toyama 1. Furthermore, this bacterium was not detected in clear types of sake from Toyama 1 (Suppl. Table 2), strongly suggesting that the *Kocuria* cells may be difficult to lyse in the sake production process.

We obtained 46 isolates from 6 *Hatsuzoe* samples of Brewery Toyama 1. Inferred from the 46 partial 16S rDNA sequences, 23 isolates (from 6 *Hatsuzoe* samples), 12 isolates (from 4 *Hatsuzoe* samples), 6 isolates (from 3 *Hatsuzoe*

samples), 2 isolates (from 1 *Hatsuzoe* sample), 2 isolates (from 1 *Hatsuzoe* sample), and 1 isolate had similar sequences to the genera *Kocuria*, *Staphylococcus*, *Bacillus*, *Leifsonia*, *Microbacterium*, and *Enterococcus*, respectively (Table 3). The isolates of *Kocuria* were obtained from all of the 6 *Hatsuzoe* samples, strongly suggesting that these isolates may be contaminated from the environments of Brewery Toyama 1. The DNA from the genus *Kocuria* was detected in the sakes of Brewery Toyama 1 but was not detected in other breweries (Suppl. Table 2). Thus, those *Kocuria* isolates may be inhabited in Brewery Toyama 1.

Cluster 1 consisted of 33 samples, which is the largest cluster. However, no genera were detected across all 33 samples (Suppl. Table 2). The most frequently detected genus was *Staphylococcus*, detected in a range between 1.2% of sample E01 and 97.3% of sample F2, and in 17 samples altogether (Suppl. Table 2), (Fig. 3). Interestingly, *Staphylococcus* was detected in all 11 samples of *sake-kasu* (Suppl. Table 2), (Fig. 3). Among the *sake-kasu* samples, the

percentage of *Staphylococcus* exceeded 50% in the samples F1, F2, F3, F9, F10, and F11 (Suppl. Table 2), (Fig. 3).

The variation of sequences detected in *sake-kasu* samples was lower than those of clear and cloudy sakes Fig. (3). Twelve different genera were detected in the *sake-kasu* samples (Suppl. Table 2), of which 11 were also detected in clear and cloudy sake samples (Suppl. Table 2). The genus *Collimonas* was only detected in the *sake-kasu* sample, F8 (1.4%) (Suppl. Table 2). *Collimonas* is capable of extracting nutrients from living fungi and from rocks and minerals [16], and its presence strongly suggests that bacterial DNA detected in *sake-kasu* may come from dead bacterial cells present, together with yeast cells, in the sediment. In sake solutions, extracellular bacterial DNA may exist from when bacterial cells lyse during the production process. The DNA from unlysed cells is also included in *sake-kasu* after filtration. Thus, the microbiome in *sake-kasu* may be a part of that in sake solutions (Fig. 3).

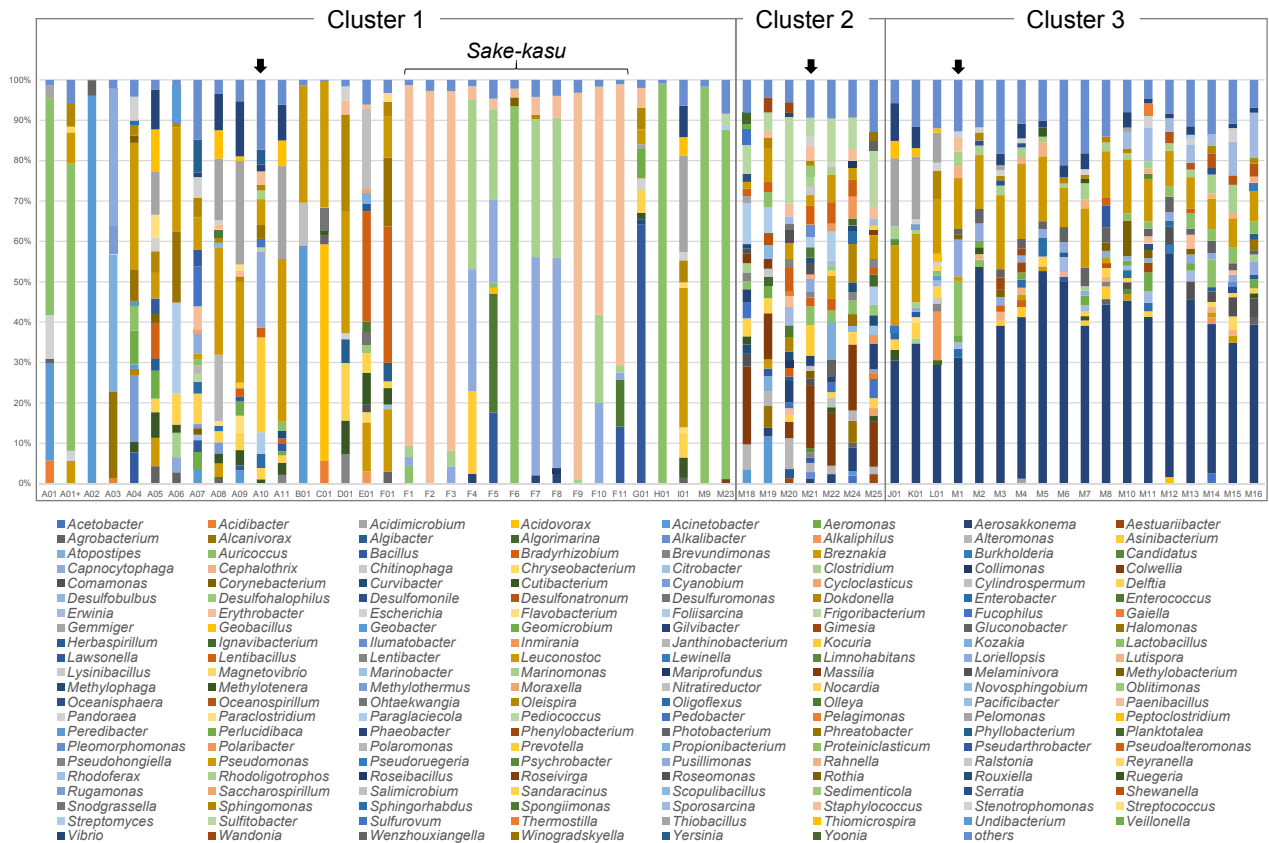


Fig. (3). Comparison of bacterial DNA components at the genus level. Cloudy sakes (arrows) and *sake-kasu* samples are indicated. The others are clear sakes.

**Table 3. Bacterial isolates from *Hatsuzoe* samples.**

<i>Hatsuzoe</i>	Sampling Date	Isolate	Genus
Sample 1	23-Oct-18	MB1023_1	<i>Bacillus</i>
		LB1023_2	<i>Kocuria</i>
		TGY1023_2	<i>Kocuria</i>
		TGY1023_4_1	<i>Leifsonia</i>
		TGY1023_4_2	<i>Leifsonia</i>
		LB1023_3	<i>Staphylococcus</i>
Sample 2	13-Nov-18	TGY1113_1_15	<i>Bacillus</i>
		LB1113_2	<i>Kocuria</i>
		MB1113_2	<i>Kocuria</i>
		TGY1113_2	<i>Kocuria</i>
Sample 3	20-Nov-18	LB1120_1_15	<i>Enterococcus</i>
		LB1120_1_30	<i>Kocuria</i>
		LB1120_2	<i>Kocuria</i>
		MB1120_1	<i>Kocuria</i>
		TGY1120_1	<i>Kocuria</i>
		TGY1120_3	<i>Kocuria</i>
		TGY1120_1_15	<i>Microbacterium</i>
		TGY1120_2_15	<i>Microbacterium</i>
Sample 4	27-Nov-18	MB1120_2	<i>Staphylococcus</i>
		LB1127_2	<i>Kocuria</i>
		LB1127_3	<i>Kocuria</i>
		MB1127_2	<i>Kocuria</i>
		TGY1127_2	<i>Kocuria</i>
Sample 5	4-Dec-18	TGY1127_3	<i>Staphylococcus</i>
		LB1204_1	<i>Kocuria</i>
Sample 6	18-Dec-18	MB1218_3_30_1	<i>Bacillus</i>
		MB1218_3_30_2	<i>Bacillus</i>
		TGY1218_4_1	<i>Bacillus</i>
		TGY1218_4_2	<i>Bacillus</i>
		LB1218_1_15	<i>Kocuria</i>
		LB1218_1_30	<i>Kocuria</i>
		LB1218_2_30	<i>Kocuria</i>
		MB1218_1_15	<i>Kocuria</i>
		MB1218_2_30	<i>Kocuria</i>
		MB1218_5_30	<i>Kocuria</i>
		TGY1218_1_15	<i>Kocuria</i>
		TGY1218_1_30	<i>Kocuria</i>
		LB1218_2_15	<i>Staphylococcus</i>
		LB1218_3_30	<i>Staphylococcus</i>
		MB1218_2_15	<i>Staphylococcus</i>
		MB1218_4_1	<i>Staphylococcus</i>
		MB1218_4_2	<i>Staphylococcus</i>
		TGY1218_2_15_1	<i>Staphylococcus</i>
TGY1218_2_15_2	<i>Staphylococcus</i>		
TGY1218_2_30	<i>Staphylococcus</i>		
TGY1218_3_30	<i>Staphylococcus</i>		

We also pose that there are certain bacteria that are difficult to lyse during sake production. *Staphylococcus*, *Loriellopsis*, and *Rhodoligotrophos* are likely candidates. Conversely, some bacteria are likely to lyse easily, with their DNA detected in sake solutions, but not detected in *sake-kasu*, and are likely to include *Pseudomonas* and *Delftia*. The

structure of a bacterial cell surface is likely related to its resistance to lysis from exposure to ethanol. The bacterial diversity in cloudy sake is higher than in *sake-kasu*, indicating that the genetic diversity of lysed bacterial cells is more diverse than that of unlysed, dead bacteria in sake sediment.

In cluster 1, the percentage of *Lactobacillus* exceeded 50% in samples A01, A01+, H01, F6, M9, and M23 (Suppl. Table 2), (Fig. 3). *Lactobacillus* is used in fermentation starter production [1, 2, 5]. In addition, some species of *Lactobacillus* are known to spoil sake [17, 18]. Strains known to cause spoilage were detected in samples A01, A01+, H01, M9, and M23 (Suppl. Table 1). On the other hand, in the sample F6, *Lactobacillus sakei* that does not cause spoilage was obtained (Suppl. Table 1).

Cluster 2 consisted of 6 types of clear sakes and one type of cloudy sake (Fig. 2), and is the smallest cluster but with the highest diversity (91 genera) (Suppl. Table 2), (Fig. 3). The genera *Alteromonas*, *Colwellia*, *Pseudoalteromonas*, *Pseudomonas*, and *Sulfitobacter* were detected in all seven samples. The most detected genera in samples M18, M19, M20, M21, M22, M24, and M25 were *Colwellia* (19%), *Acinetobacter* (11.7%), *Sulfitobacter* (21.4%), *Colwellia* (15.6%), *Colwellia* (13.1%), *Colwellia* (16.3%), and *Sulfitobacter* (13.8%), respectively (Table 3).

Cluster 3 consisted of 17 samples of clear sake and one sample of cloudy sake (Fig. 2). *Aero sakkonema* and *Pseudomonas* were detected in all 18 samples (Suppl. Table 2). *Aerosakkonema* was detected in the range of 29.4% of sample L01 and 55.4% of sample M12 (Suppl. Table 2), Fig. (3), with the species, *Aerosakkonema funiforme*, detected, which is a cyanobacterium containing intracellular gas vesicles [19]. *Pseudomonas* was detected in a range of between 7.1% of sample M15 and 20.0% of sample J01 (Suppl. Table 2), (Fig. 3).

## CONCLUSION

Bacterial DNA frequently detected in sake and *sake-kasu* samples correspond to bacteria commonly present in the environment. Bacterial contamination occurs early in the sake production process; however, bacterial cell death usually occurs on exposure to ethanol produced as the sake yeast cells grow. After bacterial cell death, if bacterial cells lyse, the DNA is released into the sake solution, but if the cells do not lyse, they will precipitate toward the sediment. Most contaminating bacterial cells lyse during the production process; therefore, while both the microbiome in cloudy and clear sake is diverse, there is less microbial diversity in *sake-kasu*. In this study, *Pseudomonas* and *Deftia* were frequently detected in sake samples but were not detected in *sake-kasu*. Thus, it is likely that these bacteria lyse easily during sake production.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## HUMAN AND ANIMAL RIGHTS

No animals/humans were used in the study that is the basis of this research.

## CONSENT FOR PUBLICATION

Not applicable.

## AVAILABILITY OF DATA AND MATERIALS

Not applicable.

## CONFLICTS OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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None.

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## SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Website along with the published article.

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