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Evaluating the technological properties of lactic acid bacteria in Wagyu cattle milk

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Research Article

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Abstract

This paper reveals the technological properties of lactic acid bacteria isolated from raw milk (colostrum and mature milk) of Wagyu cattle raised in Okayama Prefecture, Japan. Isolates were identified based on their physiological and biochemical characteristics as well as 16S rDNA sequence analysis. *Streptococcus lutetiensis* and *Lactobacillus plantarum* showed high acid and diacetyl-acetoin production in milk after 24 h of incubation at 40 and 30°C, respectively. These strains are thought to have potential for use as starter cultures and adjunct cultures for fermented dairy products.

Lactic acid bacteria (LAB) are used in many fermented foods, particularly fermented dairy products, such as cheese, buttermilk, and fermented milk, in which the products of LAB, such as lactic acid and diacetyl/acetoin, contribute to flavour, texture, and prolongation of shelf life. Generally, LAB inhabit nutrient-rich niches, such as raw milk. Numerous LAB, including *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Enterococcus casseliflavus*, *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans*, *Pediococcus acidilactici*, *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *L. helveticus*, *L. plantarum*, *L. casei*, *L. brevis*, *L. amylophilus*, and *L. rhamnosus*, have been isolated from raw milk of some mammalian species, including cows, goats, sheep, and camels (Badis *et al.*, 2004; Khedid *et al.*, 2009; McAuley *et al.*, 2015; Castro *et al.*, 2016; Abushelaibi *et al.*, 2017). Although dairy cow milk has been investigated, studies on the raw milk of beef cattle, such as Wagyu cattle, are limited.

Wagyu cattle is known as a beef breed, and the milk of beef cattle is not generally used commercially. However, some cheeses in the world are made from the milk of beef cattle, such as Serrano cheese, which is made from the raw milk of Criollo cattle in Brazil (Vitrolles, 2011). Milk from beef cattle is an unused resource, and investigations into the application of the milk for fermented dairy products may contribute to sustainable meat and dairy industries. LAB in raw milk are thought to contribute to the fermentation and the texture of this cheese, and beef cattle milk and its LAB complement make it unique among the world's cheeses. In contrast, raw milk harbours many microorganisms, including food poisoning bacteria, such as *Listeria monocytogenes* and *Staphylococcus aureus*, and food spoilage bacteria, such as *Bacillus, Escherichia*, and *Pseudomonas*. It may be difficult to ensure the safety of the cheese without pasteurization (Rosa *et al.*, 2008; Tiwari *et al.*, 2015). The efficient pasteurization of milk (low temperature/long time; 63°C for 30 min) should eliminate the risk from food poisoning and spoilage bacteria, whilst cheese can be made safely with raw milk in dairy developed countries (Little *et al.*, 2008). The milk for cheese-making can be pasteurized to ensure the safety of the cheese, however, indigenous LAB are also killed. Therefore, starter or non-starter LAB should be isolated from the raw milk and reinoculated in the milk after pasteurization.

It is important for starter LAB to exhibit rapid lactic acid production and/or aroma production for stiff curd and to suppress the growth of spoilage bacteria to obtain the desired flavour. LAB cultures must have Generally Regarded as Safe (GRAS) status and also meet a number of good technological properties, e.g., easy propagation and incorporation into foods, long term survival and safety in food products, and clinically validated and documented health effects. In this study, we isolated and identified LAB from the raw milk of Wagyu cattle raised in Japan. Then, the technological properties of the LAB were investigated for usage as a starter or as an adjunct culture for fermented dairy products.

Materials and methods

Sampling

Eighteen raw milk samples were collected from 18 Japanese Black Wagyu cattle at the Okayama Prefectural Center for Animal Husbandry & Research between 2015 and 2018. Samples were obtained by hand milking. Thirteen samples were obtained from mature milk, and the other five samples were colostrum. Samples were collected into sterilized bottles,

and the bottles were transported to the laboratory in a cooler box (approximately 10°C). Then, the samples were immediately utilized in an enumeration test.

Enumeration and isolation

Serial dilutions were made of each of the samples (1 ml) in a sterile 0.85% NaCl solution (9 ml), and these dilutions were poured into the Plate Count Agar with Bromocresol purple (BCP agar, Nissui, Japan) containing cycloheximide (10 mg/l). The plates were incubated aerobically at 30°C for 5 d. Following the incubation, the colonies with yellowish peripheries were enumerated, and then colonies were randomly selected and purified by streaking.

The strains were incubated in tryptone, yeast extract, lactose and glucose (TYLG) broth (tryptone: 10 g/l; yeast extract: 5.0 g/l; lactose 5.0 g/l: glucose: 5.0 g/l: Tween 80: 1.0 g/l; and L-cysteine HCl monohydrate: 0.1 g/l; pH 6.8 ± 0.2) and stocked in 10% reconstituted skim milk at −20°C.

Phenotypic identification of raw milk isolates

The isolated strains were identified based on their physiological and biochemical characteristics as described by de Vos *et al.* (2009) and Wood and Holzapfel (1995).

The tests included Gram staining, a catalase test, a growth temperature test, the production of gas from glucose, the type of lactic acid isomers, NH_3 production from arginine and carbohydrate (22 sugars) fermentation. The growth temperature test was conducted for up to 7 d of incubation in TYLG broth containing 0.006% BCP. The production of gas from glucose was tested in the medium (tryptone: 10 g/l; yeast extract: 5.0 g/l; glucose: 50 g/l; Tween 80: 1.0 g/l; L-cysteine HCl monohydrate: 0.1 g/l; and manganese sulphate: 0.04 g/l; pH 6.8 ± 0.2) with a Durham fermentation tube. The types of lactic acid isomers produced from glucose were assayed by high-pressure liquid chromatography equipped with a Sumichiral OA-5000 column (Sumika, Japan) (Otsuka *et al.*, 1994). The carbohydrate fermentation profile of all strains was determined as follows. Individual sugar solutions were prepared at 5.0% (w/v) except esculin, which was a 2.5% (w/v) solution, and the solutions were sterilized using a 0.22-µm membrane filter (Sartorius, Minisart, Germany). Then, 0.5 ml of the sterile filtrate sugar was added to 4.5 ml of autoclaved basal medium (tryptone: 10 g/l; yeast extract: 5.0 g/l; Tween 80: 1.0 g/l; L-cysteine HCl monohydrate: 0.1 g/l; and BCP: 60 mg/l; pH 6.8 ± 0.2). The test strain was subcultured in 5 ml of TYLG broth at 30°C for 24–48 h, and the culture was centrifuged (1000 × ***g***; 10 min). The cells were washed with 5 ml of sterile 0.85% NaCl solution, and 50 µl of this cell suspension was inoculated into 5 ml of 22-sugar medium. The BCP colour change in the medium caused by acid production was observed every day for 7 d of incubation at 30°C.

Genetic identification

The strains were also identified by 16S rDNA sequence analysis. Total DNA was extracted from bacterial strains for 16S rDNA gene analysis (Reyes-Gavilan *et al.*, 1992). The partial 16S rRNA gene was amplified by PCR using Takara EX Taq (Takara Bio, Shiga, Japan). The bacteria-specific primer sequences were 5′-GTTTGATCCTGGCTCA-3′ (10F) and 5′-TACCAGGGTATC TAATCC-3′ (800R), and PCR was performed in 30 cycles (30 s at 94°C, 60 s at 55°C and 60 s at 70°C). PCR products were purified with phenol extraction and ethanol precipitation. The purified fragments were sequenced. Sequencing reactions of the purified fragments were performed in a Bio-Rad DNA Engine Dyad PTC-220 Peltier thermal cycler using an ABI BigDye Terminator v3.1 cycle sequencing kit with AmpliTaq DNA polymerase (FS enzyme, Applied Biosystems, CA, USA). The fluorescently labelled fragments were purified with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems), and the obtained sequences were analysed using the BLAST search programme.

Lactic acid and diacetyl-acetoin production in milk

Lactic acid production was quantified by titratable acidity, and diacetyl-acetoin production was determined in accordance with the colorimetric method of Mattessich and Cooper (1989). Reconstituted skim milk (RSM) (10% wt/wt) (Snow Brand Milk Products Co., Ltd., Tokyo, Japan) was sterilized at 110°C for 20 min. The LAB strain was subcultured in TYLG broth. The activated culture was centrifuged (1,500 × ***g***, 10 min), and the cells were washed twice with an equal volume of sterile 0.85% NaCl solution. The cell suspension was inoculated into the RSM, and 1% inoculum was used in these tests. The RSM was incubated at 30 and 40°C. Titratable acidity was determined at 0, 12 and 24 h, and the diacetyl-acetoin concentration was measured at 0 and 24 h. All tests were performed in triplicate.

Statistical analysis

Significant differences between the viable cell counts of mature milk and colostrum were determined by Student's *t*-test ($P < 0.05$) using Excel 2019 (Microsoft, Redmond, WA, USA). To identify the differences in the lactic acid and diacetyl-acetoin production, one-way analysis of variance (ANOVA) was applied to the means, and significant differences between the means were determined by the Student-Newman-Keuls test ($P < 0.01$) applied using Statview 5.0 software (SAS Institute, Cary, NC, USA).

Results

Enumeration of viable cell counts

Viable cells in eighteen raw milk samples of Wagyu cattle were enumerated on BCP agar plates (Table 1). The viable cell counts of all samples ranged from 1.0 to 5.0 log cfu/ml with an average of 2.7 log cfu/ml. The average viable cell count in mature milk was 2.5 log cfu/ml, and the average in colostrum was 3.4 log cfu/ml. No significant differences were noted between the viable cell counts of mature milk and colostrum ($P < 0.05$).

Phenotypic and genetic identification of lactic acid bacteria

LAB were detected in 9 samples (sample nos. 1, 4, 6, 7, 12, 15–18): 5 mature milk and 4 colostrum samples. Almost all of the common LAB genera, such as *Lactobacillus, Streptococcus, Lactococcus, Leuconostoc, Pediococcus,* and *Enterococcus*, can grow on BCP agar plate. Colonies on the BCP agar of the other samples were streaked and were identified as *Aerococcus*, *Micrococcus*, *Staphylococcus*, yeasts and other catalase-positive bacteria from the identification tests. Fifteen LAB strains were obtained from the raw milk and

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Table 1. Viable cell counts on BCP plate count agar of raw milk from Wagyu cattle

Sample no.	Sampling date	Type of milk	viable cell counts (log cfu/ml)	Average	
1	2015.8	Mature	2.6		
2	2015.12	Mature	2.3		
3	2016.5	Mature	2.0	2.4	
4	2016.5	Mature	1.0		
5	2018.9	Mature	4.2		
6	2016.5	Colostrum	2.8		
7	2017.11	Colostrum	3.7	3.4	
8	2017.11	Colostrum	3.6		
9	2017.11	Colostrum	3.4		
10	2015.8	Mature	1.0		2.7
11	2015.8	Mature	1.0		
12	2015.12	Mature	1.0		
13	2016.5	Mature	1.0		
14	2018.5	Mature	3.6	2.5	
15	2018.5	Mature	2.3		
16	2018.5	Mature	5.0		
17	2018.9	Mature	4.2		
18	2018.9	Mature	3.4		

colostrum samples from Wagyu cattle. Seven strains were rods, and eight were cocci. Table 2 shows the characteristics of the isolated LAB.

One strain was identified as *L. fermentum* and was isolated from mature milk (sample no. 1). The strain produced gas from glucose and exhibited growth at 45°C and no growth at 15°C. The strain produced NH_3 from arginine and fermented galactose, maltose, melibiose, raffinose, ribose, and sucrose but not esculin and melezitose. The 16S rDNA sequence revealed 99.74% homology to *L. fermentum* (accession no.: LC500973).

Three strains were identified as *L. plantarum* and were isolated from mature milk (sample nos. 1, 4, and 7). These strains exhibited growth at 15°C but not at 45°C and produced no gas from glucose. These strains produced DL-lactic acid, and the strains fermented almost all the tested sugars. The 16S rDNA sequences showed 99.44, 99.87, and 99.87% homology to *L. plantarum* (accession nos.: LC379422, LC500974, and LC500975, respectively).

Three strains were identified as *L. brevis* and were isolated from colostrum (sample no. 15). These strains produced gas from glucose, exhibited growth at 15°C but not at 45°C and produced NH_3 from arginine and fermented maltose, melibiose, and ribose, but not melezitose. The 16S rDNA sequences showed 98.94, 98.97, and 99.21% homology to *L. brevis* (accession nos.: LC500976, LC500977, and LC500978, respectively).

Three strains were identified as *S. pluranimalium* and were isolated from mature milk (sample nos. 4, 6, and 7). The strains produced L-lactic acid and did not produce gas from glucose. These strains fermented fructose and glucose, and the 16S rDNA sequences showed 99.46, 99.41, and 99.33% homology to *S. pluranimalium* (accession nos.: LC566144, LC379421, and LC566145, respectively).

One strain was identified as *S. lutetiensis* and was isolated from mature milk (sample no. 12). The strain produced L-lactic acid and did not produce gas from glucose. The strain fermented lactose, maltose, raffinose, and sucrose but not arabinose, mannitol, melezitose, ribose, and sucrose. The 16S rDNA sequence showed 99.86% homology to *S. lutetiensis* (accession no.: LC500982).

One strain was identified as *E. pseudoavium*, and it was isolated from colostrum (sample no. 16). This strain exhibited growth at 10°C and 45°C and produced L-lactic acid. However, the strain did not produce NH_3 from arginine or gas from glucose. Additionally, this strain fermented cellobiose, maltose, mannitol, ribose, sorbitol, and trehalose but not melezitose, melibiose, raffinose, rhamnose, and sucrose. The 16S rDNA sequence showed 99.74% homology to *E. pseudoavium* (accession no.: LC500979).

Two strains were identified as *E. villorum* and were isolated from colostrum (sample nos. 17 and 18). These strains exhibited growth at 10°C and 45°C, produced L-lactic acid and did not produce gas from glucose. The strains fermented amygdalin, cellobiose, fructose, galactose, lactose, maltose, mannose, and ribose but not arabinose, mannitol, melezitose, rhamnose, and sorbitol. The 16S rDNA sequence showed 99.09 and 99.60% homology to *E. villorum* (accession nos.: LC500981 and LC566146).

One strain was identified as *Leu. mesenteroides* subsp. *dextranicum* and was isolated from colostrum (sample no. 16). The strain exhibited growth at 10°C and 45°C and produced D-lactic acid and gas from glucose. The strain fermented fructose, lactose, maltose, ribose, sucrose, and trehalose but not arabinose. The 16S rDNA sequence showed 99.47% homology to *Leu. mesenteroides* subsp. *dextranicum* (accession no.: LC500980).

Lactic acid production in milk

Lactic acid production in milk was measured with all strains isolated from raw Wagyu cattle milk. Figure 1 shows the lactic acid production of *S. lutetiensis* PUHM1034, *L. plantarum* PUHM1023, and *L. plantarum* PUHM1026. *S. lutetiensis* PUHM1034 produced approximately 0.6% lactic acid in milk when incubated for 24 h at 40°C. These levels were followed by *L. plantarum* PUHM1023 and PUHM1026, which produced 0.288 and 0.242% lactic acid at 30°C, respectively. The other LAB rarely produced lactic acid in milk even after 24 h of incubation (data not shown).

Diacetyl-acetoin production in milk

Figure 2 shows the diacetyl-acetoin production of LAB isolated from raw Wagyu cattle milk. *S. lutetiensis* PUHM1034, *L. plantarum* PUHM1026, and *E. villorum* PUHM1033 produced significantly higher diacetyl/acetoin levels than the other LAB. *S. lutetiensis* PUHM1034 produced 4.78 mg/l diacetyl-acetoin in milk when incubated for 24 h at 30°C followed by *L. plantarum* PUHM1026 (2.06 mg/l) and *E. villorum* PUHM1033 (2.02 mg/l) at 30°C.

Discussion

The average viable cell count in Wagyu cattle milk obtained in this study was consistent with that in previous reports. The average viable cell count in raw milk of Holstein-Friesian cows was

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Table 2. Taxonomic properties of lactic acid bacteria isolated from the raw milk of Wagyu cattle

								wm1604		sm1620					
Strain	PUHM1024	PUHM1025	PUHM1026	PUHM1023	PUHM1027	PUHM1028	PUHM1029	PUHM1035	PUHM1022	PUHM1036	PUHM1034	PUHM1030	PUHM1032	PUHM1033	PUHM1031
Isolated sample No.	1	1	4	7	15	15	15	4	6	7	12	16	17	18	16
16S rDNA sequence	*Lactobacillus fermentum*	*Lactobacillus plantarum*	*Lactobacillus plantarum*	*Lactobacillus plantarum*	*Lactobacillus brevis*	*Lactobacillus brevis*	*Lactobacillus brevis*	*Streptococcus pluranimalium*	*Streptococcus pluranimalium*	*Streptococcus pluranimalium*	*Streptococcus lutetiensis*	*Enterococcus pseudoavium*	*Enterococcus villorum*	*Enterococcus villorum*	*Leuconostoc mesenteroides* ssp. *dextranicum*
16S rDNA sequence homology (%)	99.74	99.44	99.87	99.87	98.94	98.97	99.21	99.46	99.41	99.33	99.86	99.74	99.09	99.60	99.47
Accession No.	LC500973	LC379422	LC500974	LC500975	LC500976	LC500977	LC500978	LC566144	LC379421	LC566145	LC500982	LC500979	LC500981	LC566146	LC500980
Cell shape	rod	rod	rod	rod	rod	rod	rod	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci
Growth at															
10 °C	–	ND	ND	ND	–	–	–	–	–	–	–	+	+	+	+
15 °C	–	+	+	+	+	+	+	+	+	+	+	ND	ND	ND	+
40 °C	ND	+	+	+	+	+	+	ND	ND	ND	ND	ND	ND	ND	+
45 °C	+	–	–	–	–	–	–	+	+	+	+	+	+	+	–
NH_3 from arginine	+	–	–	–	+	+	+	–	–	+	–	–	+	+	–
Gas from glucose	+	–	–	–	+	+	+	–	–	–	–	–	–	–	+
Lactic acid isomer	DL	DL	DL	DL	DL	DL	DL	L	L	L	L	L	L	L	D
Acid detected (No. + isolates)															
Amygdalin	–	+	+	+	–	–	–	+	+	–	+	–	+	+	+
D-Arabinose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D-Cellobiose	+	+	+	+	+	+	–	+	+	–	+	+	+	+	+
Esculin	–	+	+	+	+	+	–	+	+	–	+	–	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Lactose	+	+	+	+	+	–	–	–	+	–	+	–	+	+	+
D-Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannitol	–	+	+	+	–	–	–	+	+	–	–	+	–	–	+
D-Mannose	+	+	+	+	+	+	–	+	+	–	+	+	+	+	+
D-Melezitose	–	+	+	+	–	–	–	+	+	–	–	–	–	–	–
D-Melibiose	+	+	+	+	+	+	+	–	–	+	+	–	+	+	+

(*Continued*)

Table 2. (*Continued.*)

Strain	wm1604							sm1620							
	PUHM1024	PUHM1025	PUHM1026	PUHM1023	PUHM1027	PUHM1028	PUHM1029	PUHM1035	PUHM1022	PUHM1036	PUHM1034	PUHM1030	PUHM1032	PUHM1033	PUHM1031
Na-Gluconate	+	+	+	+	+	−	+	−	−	+	−	−	−	−	+
L-Raffinose	+	+	+	+	+	−	−	−	−	−	+	−	−	−	+
L-Rhamnose	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−
D-Ribose	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+
Salicin	−	+	+	+	+	−	+	+	+	−	+	−	+	+	+
D-Sorbitol	-	−	−	−	−	−	−	−	−	−	−	+	−	−	−
Sucrose	+	+	+	+	+	−	−	+	+	−	+	−	−	−	+
D-Trehalose	+	+	+	+	+	+	+	+	+	−	+	+	−	−	+
D-Xylose	+	−	+	+	+	−	+	−	−	+	−	−	+	+	+

Symbols: +, positive; −, negative. ND, not detected.

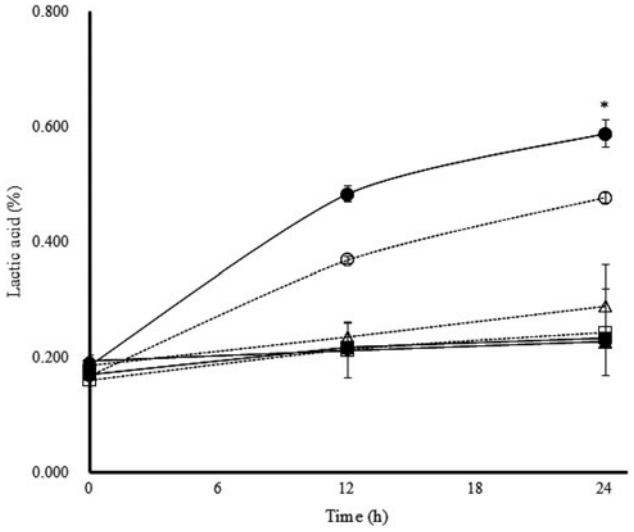

Fig. 1. Lactic acid production in milk at 30°C (open symbols and dashed lines) and 40°C (closed symbols and solid lines). Circles: *S. lutetiensis* PUHM 1034; triangles: *L. plantarum* PUHM 1023; squares: *L. plantarum* PUHM 1026. *Statistically significant difference ($P < 0.01$).

reported as approximately 3 log cfu/ml (Franciosi *et al.*, 2009; Espeche *et al.*, 2012; Mallet *et al.*, 2012). We collected all samples from Wagyu cattle raised at the Okayama Prefectural Center for Animal Husbandry & Research. The feed and rearing environment of all Wagyu cattle were the same. Therefore, it can be hypothesized that these factors had minimal influence on the viable cell counts and the diversity of LAB in this investigation.

The species isolated in this study have been isolated from raw milk of some mammalian species (Badis *et al.*, 2004; Khedid *et al.*, 2009; McAuley *et al.*, 2015; Castro *et al.*, 2016; Guccione *et al.*, 2016; Abushellaibi *et al.*, 2017; Fugl *et al.*, 2017). We previously reported that various types of LAB were isolated from the raw milk of Wagyu cattle (Tsuda, 2015). Among them, only *L. plantarum* was isolated in common with the previous study. In this study, *L. plantarum* and *S. pluranimalium* were isolated from multiple mature milk samples, and *E. villorum* was isolated from multiple colostrum samples. However, no species were isolated from both the mature milk and colostrum samples of Wagyu cattle. It is possible that LAB in colostrum differ from those in mature milk. This is the first report about LAB in the colostrum of Wagyu cattle. Further work is needed to investigate the distribution of LAB in raw milk.

L. brevis PUHM1029, *S. pluranimalium* PUHM1035 and PUHM1036, and *E. pseudoavium* PUHM1030 fermented galactose but not lactose, and *L. brevis* PUHM1028 did not ferment either. This finding indicates that these LAB strains rely on other microorganisms that can break down lactose into glucose and galactose outside the cell in milk for carbon sources. Indeed, these strains did not show acid production in milk on single use (Table 2).

The main role of starter cultures is acid production to form a gel matrix in milk, and another role is flavour contribution to fermented dairy products. *S. lutetiensis* PUHM1034 and *L. plantarum* PUHM1026 are potential candidates for starter or adjunct culture of fermented dairy products based on this fundamental technological characterization. Further work is needed to identify specific bio-active peptides and volatile fatty acids with LC-MS and GC-MS, respectively.

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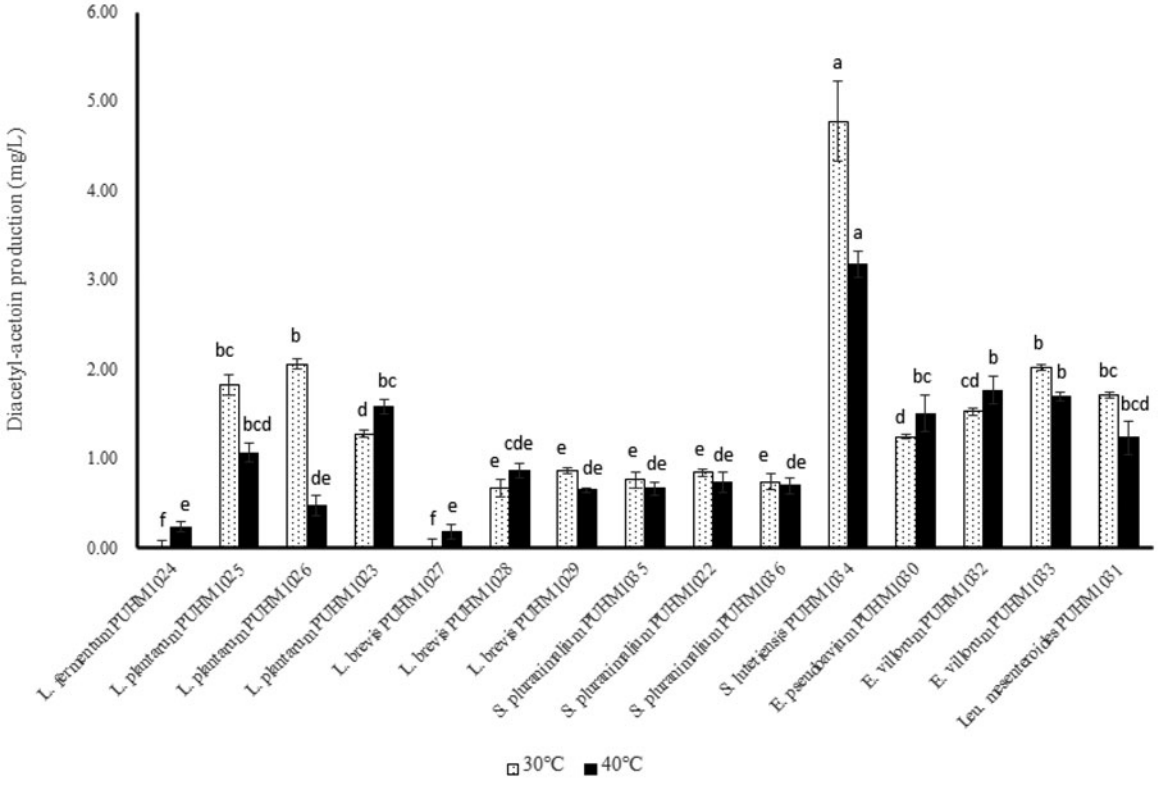

Fig. 2. Diacetyl-acetoin production in milk at 30°C and 40°C. Different letters indicate statistically significant differences ($P < 0.01$) at 30°C and 40°C, respectively.

The titratable acidity of fermented milk products, such as yogurt, is approximately 0.7% in Japan. The PUHM1034 strain will cause gel formation in milk by acid production after 24 h of incubation. The formation of lactic acid results in a reduction in the surface charge on a casein micelle from the negative charge at pH neutral, and the net charge becomes zero at the isoelectric point (pH 4.6) of casein. This change in surface charge allows casein micelles to aggregate, and the aggregation results in a gel being formed at pH ~ 5.3 (Robinson *et al.*, 2006). In this paper, some LAB strains produced lower concentrations of lactic acid. These strains cannot be used to make an acid milk. However, LAB that produce lower concentrations can be used for some cheese.

Diacetyl-acetoin production was higher with strains PUHM1034 and PUHM1026 compared with previous reports. Badis *et al.* (2004) reported that the LAB strain isolated from raw goat's milk produced 1.02 mg/L diacetyl-acetoin, and Beshkova and his coworkers reported that a yogurt starter culture of *S. thermophilus* 15a and *L. bulgaricus* 1–9 alone and together produced 0.33, 1.85, and 2.2 mg/l diacetyl-acetoin in milk, respectively (Beshkova *et al.*, 1998). However, the diacetyl-acetoin production by strains PUHM1036 and PUHM1026 was much less than that reported by De Leonardis *et al.* (2013), who reported that *L. rhamnosus* produced 27.6 mg/l diacetyl-acetoin in milk. Diacetyl/acetoin is produced by some LAB during citrate fermentation. Two metabolic pathways for the production have been considered. In one pathway, pyruvate and citrate metabolism in *Lc. lactis* subsp. *lactis* biovar *diacetylactis* is thought to occur given that diacetyl is formed by chemical oxidative decarboxylation of acetolactic acid (Monnet *et al.*, 1994; Tamime *et al.*, 2006). In the second pathway, the direct synthesis of diacetyl from acetyl-CoA has been postulated; however, the diacetyl synthase enzyme has never been directly isolated from lactic acid bacteria.

S. lutetiensis was also isolated from camel milk, and its isolates were fast acidifiers in camel and bovine milk (Fugl *et al.*, 2017). *S. lutetiensis* exhibits good potential as a starter culture for acid production in milk, and this species might be able to be substituted for the traditional yogurt starter *Streptococcus thermophilus*. The largest obstacle to its use is that the safety and/or pathogenicity of *S. lutetiensis* have not been determined to date. *S. lutetiensis* belongs to the *Streptococcus* 'bovis/equinus' complex (de Vos *et al.*, 2009). This group represents a collection of streptococci of human and animal origin, and its classification is currently under revision using molecular data. The species in this group are often isolated from horse faeces, bovine faeces, mastitis, human faeces, and human clinical sources. Jin *et al.* (2013) sequenced the entire genome of *S. lutetiensis* strain 033, and a putative pathogenic island was identified. Putative virulence genes detected in the genome of *S. lutetiensis* included pneumococcal cell surface adherence protein A, laminin-binding protein, pilus-associated adhesin, sortase A, streptococcal lipoprotein rotamase A, streptococcal enolase, pneumococcal surface antigen, C3-degrading protease, serine protease, and trigger factor. In addition, a haemolytic toxin gene was identified in the *S. lutetiensis* genome that activated the neutrophil signalling pathways in the brain endothelium. In addition, some strains of this group are used for traditional food fermentations (Fugl *et al.*, 2017) and may display traits associated with safety. On the other hand, *L. plantarum* is a GRAS microorganism.

In conclusion, we were able to isolate and identify various LAB from Wagyu milk. *S. lutetiensis* PUHM 1036 has traits suitable for a starter culture. However, the safety of this species is unconfirmed, therefore, safety tests are required before its use. Although *L. plantarum* PUHM1026 exhibited weak acid

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productivity, this strain is generally recognized as safe and showed high aroma production. This strain will be used as an adjunct culture in fermented dairy products. In addition, these strains will be further evaluated for the aroma formed during cheese fermentation and their probiotic properties as a functional food. This paper may provide further information on dairy products from milk of beef cattle as an unused resource.

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