Effect of milk types on the attributes of a glutinous rice wine-fermented yogurt-like product

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ABSTRACT

This study was conducted to analyze the effect of milk types on the attributes of the glutinous rice wine-fermented yogurt-like product named Kouwan Lao (KWL). Four types of raw milks were used in this study, including high temperature, long time (HTLT: H milk), HTLT milk supplemented with 3% skim milk powder (S milk), pasteurized milk (P milk), and ultra-high temperature milk (U milk). Microbiological compositions of the fermented glutinous rice and KWL at different stages were analyzed using PCR-denaturing gradient gel electrophoresis and gene sequencing based on 16S rRNA and 26S rRNA. The physicochemical properties of KWL samples were determined, and textural properties of those were analyzed using a texture analyzer (Jiawei Innovation and Technology Co. Ltd., Zhejiang Province, China). The microstructure of KWL samples was observed using scanning electron microscopy. The results showed that the milk types had significant influences on the bacterial composition of KWL. In the curdling process, the predominant bacteria of H, S, P, and U KWL samples were *Lactobacillus brevis*, *Janthinobacterium* sp., *Lactobacillus casei*, and *Streptococcus agalactiae*, respectively. In the ripening process, the main strains in H KWL were *Enterococcus faecium* and *Pediococcus pentosaceus*. *Lactobacillus casei* and *Lactobacillus paracasei* were the dominant bacteria of U KWL. *Lactobacillus casei* was the main strain of P KWL, and no bacteria were detected in S KWL. *Saccharomyces cerevisiae* was the dominant fungus of KWL, and no significant effect of milk types on fungal

composition of KWL was found. The results of physicochemical properties showed that significant differences in protein contents were found in the KWL samples, and P KWL had the highest protein content. The fat content of U KWL was significantly higher than that of samples from the other 3 groups. The U KWL and P KWL showed lower moisture than that of the other 2 KWL samples. In addition, no significant difference in pH value was found in all samples. The results of texture analysis and microstructure showed that compared with other 3 types of KWL samples, the related mass parameters of U KWL were more advantageous and improved significantly with the increase of the heat treatment temperature of raw milk and the addition of skim milk powder. Our findings revealed the effects of milk types on microbial composition, physicochemical properties, textural properties, and microstructure of KWL, and provided a basic theory for the optimization and industrial production of KWL.

Key words: Kouwan Lao, milk type, bacterial and fungal diversity, texture property

INTRODUCTION

Yogurt and yogurt-like products are often consumed as healthy foods, and are a growing market in the dairy industry through the world (Matumoto-Pintro et al., 2011). To increase consumer interest in the health benefits of yogurt, a variety of food combinations have been used in production of yogurt and yogurt-like products, such as honey (Sert et al., 2017), grape seed oil (Mercan et al., 2018), and pineapple peel powder (Sah et al., 2016). In addition, some studies have also revealed the effect of additives on the fermentation performance of yogurt, including pine cone and sea buckthorn berries (Mercan and Akin, 2016; Terpou et al., 2019). The above background indicates that the development of yogurt and yogurt products is highly valued and has great potential.

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In China, the glutinous rice wine-fermented were used as a starter to produce a popular traditional yogurt-like product, called as Kouwan Lao (**KWL**). Kouwan Lao consists of soft curd appearance and a sweet-set gel induced from cow milk, which coagulated with the glutinous rice wine-fermented. It is a low-acid or non-sour-tasting yogurt-like product with slightly alcoholic flavor (Onyeneho et al., 1987). However, so far, little in-depth research has been done on KWL production and microbial structure.

For hundreds of years in China, fermented glutinous rice has utilized the yeast-ball or fungus as the starter cultured on a starch-rich substrate in a solid-state fermentation process (Su et al., 2005; Wang et al., 2008). Some strains have been found in the glutinous wine-fermented and their metabolites contained various amylolytic and proteolytic enzymes to exhibit notable milk-clotting ability and contributed to the texture of ultimate KWL (Ercolini et al., 2004; Lv et al., 2012). In addition, heat treatment and increasing TS in the raw milk could affect the development of the microbial community, the activity of milk rennet, and the texture of dairy products (Muyzer et al., 1993; Sert et al., 2017). Therefore, it is very meaningful to increase the understanding about the effect of milk types on microbiology composition of KWL for its mature large-scale industrial production.

Several rapid and reliable methods have been applied to identify strains that cannot be cultured using traditional methods; among these methods, the PCR-denaturing gradient gel electrophoresis (**DGGE**) technology was considered the most mature and commonly used, and has been widely used to reveal the microbial communities of dairy products (Flórez and Mayo, 2006). Using PCR-DGGE in combination with gene sequencing, the changes in diversity and microbial structure can be monitored in a timely manner at various stages of food products without isolation of strains (Grappin and Beuvier, 1997).

In this study, KWL was produced using different types of raw milk, including HTLT milk (**H milk**), HTLT milk with 3% skim milk power (**S milk**), pasteurized milk (**P milk**), and UHT milk (**U milk**). The PCR-DGGE in combination with gene sequencing was used to analyze the microbial communities of bacteria and fungi of KWL sample with different treatments during varying stages of fermentation. The effects of different milk types on properties such as chemical composition, textural properties, and microstructure of KWL were also assessed using the AOAC International method, texture analysis, and scanning electron microscopy, respectively.

MATERIALS AND METHODS

Glutinous Rice Fermentation

Rice wine was prepared as the exclusive starter of KWL according to the report of Onyeneho et al. (1987). Glutinous rice (Pan Fu Glutinous Rice, Liaoning Province, China) was washed and soaked in distilled water (rice:water = 1:2 by volume) for 60 min at room temperature ($22 \pm 2^\circ\text{C}$). The rice with soaking water were cooked for 20 min followed by cooling to 35°C . The starter (Angel Yeast Co. Ltd., Hubei Province, China) was added into the cooled rice, then fermented in a closed environment at 30°C for 3 d. The fermenting material was stirred up every day to wet all of the grain particles uniformly. After 3 d of fermentation, the fermented product was diluted with distilled water and re-incubated for additional 3 d followed by a filter to get the clear glutinous rice wine then and stored at 4°C for further study.

Manufacturing of KWL

Eight percent (wt/vol) of the glutinous rice wine and 8% (wt/vol) of sugar (bought from the local market) were individually added to different types of milk including H milk (raw milk was obtained from a local dairy farm and sterilized at 100°C for 15 min), S milk (West Tang Biological Technology Co., Ltd., Shandong Province, China), P milk (Jiasheng Dairy Co., Ltd., Shandong Province, China), and U milk (Mengniu Dairy Co., Ltd., Inner Mongolia Autonomous Region, China). The mixed samples were fermented at 43°C until the curd was complete. Finished KWL samples were stored at 4°C and named as H KWL (made by HTLT milk), S KWL (made by HTLT milk supplemented with 3% skim milk powder), P KWL (made by pasteurized milk), and U KWL (made by UHT milk), respectively.

PCR-DGGE Analysis

The total DNA of KWL samples were extracted using the MiniBEST Universal Genomic DNA Extraction Kit (Ver 5.0, TaKaRa, Kusatsu, Japan). The primers sequences for V3-16S rRNA and D1-26S rRNA and PCR scheme were provided by previous reports (Muyzer et al., 1993; Cocolin et al., 2002). The DGGE analysis was performed on the Dcode System apparatus (Bio-Rad, Hercules, CA) following the method of Muyzer and Smalla (1998). Samples were applied to an 8% (wt/vol) polyacrylamide gel in $1 \times \text{TAE}$ buffer (40 mM Tris HCl, pH 7.4, 20 mM sodium acetate, 1.0

mM Na₂EDTA) with a denaturing range of 35 to 60% for bacteria and 35 to 70% for fungi. Electrophoresis was performed at 20 V for 60 min and then at 130 V for 8 h at 60°C (Bio-Rad). The DNA bands were recovered and sequenced by the Beijing Genomics Institute (BGI, Beijing China). All sequences were identified in the GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>). Sequences with 97% of identity or higher were considered to represent the same species.

Physicochemical Properties

The chemical composition in terms of % moisture content (wt/wt), % fat content (wt/wt), and % protein content (wt/wt) of KWL samples made with different types of raw milk was analyzed based on the methods as stated by AOAC International (1997). The pH values of KWL samples were determined with the pH meter (model MP 220, Mettler Toledo, Barueri, SP, Brazil).

Texture Profile

The texture profile of KWL samples was performed according to previous study (Sandoval-Castilla et al., 2004) with some modifications. The texture indices of samples after standing for 30 min were measured using the texture analyzer (TA-XT plus texture analyzer, Jiawei Innovation and Technology Co., Ltd., Zhejiang Province, China). The texture analyzer was set at a pre-test speed of 2.0 mm/s, a measuring speed of 1.0 mm/s, a lateral velocity of 5.0 mm/s, a inductive force of 3.0 N, and a compression distance of 10 mm using a probe of type P/0.5. The measuring times between intervals was set at 5.0 s.

Microstructure

Scanning electron microscope observation was performed using the method reported by Sandoval-Castilla et al. (2004). The KWL samples were cut into 2 × 2.5 mm strips and fixed in 2.5% pentanediol followed by storage at 4°C for more than 90 min. Samples after rinsing 3 times were dehydrated using 50%, 70%, and 90% ethanol for 15 min, respectively, and then dehydrated 3 times with 100% ethanol for 15 min followed by rinsing with 100% butyl alcohol for 15 min, and frozen at -20°C for 30 min. The samples were dried for 4 h followed by adhering to the sample stage of scanning electron microscopy. Over the scanning electron microscopy stage, a metal film with a thickness of 100 to 150 Å was applied to the surfaces of samples by using an ion sputtering apparatus and the processed samples were prepared for inspection. The images were

observed using the scanning electron microscope S-3400N (Hitachi High-Tech Co. Ltd., Tokyo, Japan).

Statistical Analysis

For each treatment, data from independent replicate trials were obtained, and the means and the standard deviations were calculated. All data were analyzed using Duncan's multiple-range test (SPSS 17.0 software for windows, SPSS Inc., Chicago, IL). One-way ANOVA was used to assess the differences such as protein, fat, moisture contents, and pH values. Significant differences were accepted at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of Milk Types on the Bacterial Community Structure in the KWL

The DGGE analysis and gene sequencing were performed to reveal the effect of milk types on bacterial structure of KWL samples, as shown in Figure 1A and Table 1. The starters of the glutinous and glutinous rice wine were used as reference controls. The results showed significant differences in DGGE profiles of KWL prepared by different milk types, which suggested that the bacterial compositions of 4 groups KWL samples were different. Significant differences were also present in dominant strains of KWL samples. In detail, among the 4 types of raw milk, bacteria were detected only in H milk and identified as *Streptococcus agalactiae*, which indicated that in the KWL samples, the main bacteria come from glutinous rice wine. In the curdling process, the predominant bacteria of H, S, P, and U KWL samples were *Lactobacillus brevis*, *Janthinobacterium* sp., *Lactobacillus casei*, and *Streptococcus agalactiae*, respectively. In the ripening process, the main strains in H KWL were *Enterococcus faecium* and *Pediococcus pentosaceus*. *Lactobacillus casei* and *Lactobacillus paracasei* were the dominant bacteria of U KWL. *Lactobacillus casei* still was the main strain of P KWL. In addition, no predominant bacteria were found in ripening of S KWL. These results indicated a significant effect of milk types on the bacterial composition of KWL during the curd and ripening process.

Analogously, *P. pentosaceus* in glutinous rice wine has been reported as the dominant bacteria in red yeast rice wine (Lv et al., 2012). In this study, *S. agalactiae* was found in the H milk and U KWL samples in the curd process, which was similar to the previous study of Liu et al. (2012), who detected *S. agalactiae* in the traditional fermented yak milk in Tibet. *Lactobacillus*

Table 1. Identities of bands obtained from the bacterial community of Kouwan Lao (KWL) made by 4 types of milk

Band no. ¹	Most similar sequence relative (species)	GenBank accession no.	Size (bp)	Identity (%)
11, H-42	<i>Pediococcus pentosaceus</i>	JN851781.1	956	100
H-22	Uncultured bacterium	EU647636.1	579	97
H-23, U-112	<i>Streptococcus agalactiae</i>	CP012419.1	2,116,810	98
H-31	<i>Lactobacillus brevis</i>	JN863608.1	949	99
H-41	<i>Enterococcus faecium</i>	KY124655.1	1,442	99
S-51	Uncultured bacterium	HE586845.1	560	99
S-52	<i>Janthinobacterium</i> sp.	KJ923803.1	1,378	99
S-53	Uncultured bacterium	KX991389.1	460	99
S-54	Uncultured bacterium	JX543563.1	467	99
P-81, U-122	<i>Lactobacillus casei</i>	KM921936.1	430	100
P-91	<i>L. casei</i>	KM921935.1	430	100
U-121	<i>Lactobacillus paracasei</i>	HQ697671.1	535	98
U-123	<i>L. casei</i>	KM921936.1	430	98

¹11 = glutinous rice wine; H-22, H-23 = high temperature, long time (HTLT) milk; H-31 = curd of H KWL (KWL made by HTLT milk); H-41, H-42 = ripened of H KWL; S-51 to S-54 = curd of S KWL (KWL made by HTLT milk with 3% skim milk); P-81 = curd of P KWL (KWL made by pasteurized milk); P-91 = ripened of P KWL; U-112 = curd of U KWL (KWL made by UHT milk); U-121 to U-123 = ripened of U KWL.

genus was the predominant strains in the curdling stage of H and P KWL, and also was detected in other traditional fermented dairy products, such as yogurt-like product with added grape seed oil, Koumiss fermented with mare's milk, and milk bread (Rahman et al., 2009; Mercan et al., 2018; Guo et al., 2019). *Janthinobacterium* sp. detected from the curd of S KWL usually exists in soil and the environments where there may be contact with starter and raw milk (Gong et al., 2017). *Enterococcus faecalis*, as nonstarter lactic acid bacteria, was the one of main strains in ripening of H KWL, and also was found in Turkish white cheese (Ispirli et al., 2016). *Lactobacillus casei* and *L. paracasei* detected

from P and U KWL have been reported to occur in sheep yogurt (Haghshenas et al., 2015).

Effect of Milk Types on the Fungal Community Structure in KWL

As shown in Figure 1B, the diversity of fungi in KWL was lower, especially compared with that of bacteria. The gene sequencing results corresponding to the bands in the DGGE picture are given in Table 2. The results showed that the fungus was not detected in all raw milk samples, and the dominant fungi in glutinous rice wine consisted of *Clavispora lusitaniae*, *Wickerhamomyces*

Figure 1. The denaturing gradient gel electrophoresis separation patterns of PCR amplified segments of the V3 region of bacterial 16S rRNA gene (A) and fungal 26S rRNA gene (B) derived from Kouwan Lao (KWL) made from 4 types of milk. Lanes: 0 = starters of the glutinous; 1 = glutinous rice wine; H2–H4, high temperature, long time (HTLT) milk = curd of H KWL (KWL made by HTLT milk), ripened of H KWL; S5–S6 = curd of S KWL (KWL made by HTLT milk with 3% skim milk), ripened of S KWL; P7–P9 = pasteurized milk, curd of P KWL (KWL made by pasteurized milk), ripened P KWL; U10–U12, UHT milk, curd of U KWL (KWL made by UHT milk), ripened U KWL. Unnumbered bands were not identified.

Table 2. Identities of bands obtained from the fungal community of Kouwan Lao (KWL) made by 4 types of milk

Band no. ¹	Most similar sequence relative (species)	GenBank accession no.	Size (bp)	Identity (%)
01	<i>Rhizopus oryzae</i>	KT160362.1	720	99
11	<i>Wickerhamomyces anomalous</i>	JF513089.1	591	99
12, 13	<i>Clavispora lusitanae</i>	KY106934.1	851	99
31, 41, 51, 111	<i>Saccharomyces cerevisiae</i>	EU441887.1	627	100

¹01 = starters of the glutinous; 11 to 13 = glutinous rice wine; 31 = the curd of H KWL [KWL made by high temperature, long time (HTLT) milk]; 41 = ripened of H KWL; 51 = curd of S KWL (KWL made by HTLT milk with 3% skim milk); 111 = curd of U KWL (KWL made by UHT milk).

anomalous, and *Rhizopus oryzae*, which was similar to the study of Wang et al. (2008) and Thanh et al. (2016). Furthermore, *Rhizopus oryzae* as the main strain of glutinous rice wine also plays an important role in the fermentation of wine because it could secrete amylase (Wang et al., 2008; Lv et al., 2012). During the curdling process of KWL, *Saccharomyces cerevisiae* was present in H, S, and U KWL, whereas no fungus was detected in P KWL. In the ripening process, the fungus was detected only in H KWL, and identified as *S. cerevisiae*. Similarly, in previous studies, *S. cerevisiae* also was found in Japanese sake, Chinese rice wine, and Indonesian ragi (Wang et al., 2012; Li et al., 2014).

However, in general, milk types did not cause significant differences in fungal composition of KWL.

Effect of Milk Types on the Physicochemical Properties of KWL

The contents of protein, fat, moisture, and values of pH of H, S, P, and U KWL samples were measured and shown in Table 3. Significant differences ($P < 0.05$) in protein contents have been found in the KWL samples made by 4 types of milk, among them, P KWL had the highest protein content, whereas the protein content of H KWL was the lowest. The fat content of U KWL was significantly higher than that of other 3 group samples ($P < 0.05$). The U and P KWL showed lower moisture than that of other 2 KWL samples ($P < 0.05$). In addition, the difference in pH values was not significant in all samples ($P > 0.05$).

The physicochemical properties of milk can be greatly affected by the thermal sterilization parameters of raw milk. Célia et al. (2017) reported that the highest protein content was found in yogurt produced by pasteurized milk compared with UHT and raw refrigerated milk, which is agreement with our study. Because skim milk powder was added to S milk, the protein content of the S KWL was higher than H KWL. The fat content in the dairy products is reported to be related to the lipolysis of fungi, especially *Saccharomyces*, which also was detected in the finished products of H KWL. Further, the difference of pH value was not significant in all samples, which was similar to a previous study (Célia et al., 2017).

Effect of Milk Types on the Textural Properties of KWL

As shown in Table 4, the effect of milk type on the textural properties of KWL was revealed. The results showed that U and P KWL samples had higher hardness, springiness, chewiness, and resilience than H and S KWL ($P < 0.05$), and the hardness of U KWL was highest in all KWL samples. These suggest that UHT milk is more advantageous in the quality control of the texture of KWL, the quality of texture increases with the increase of heat treatment temperature of raw milk. Previous studies have shown that heat treatment of raw milk, fat, and protein content could influence the textural properties of dairy products, especially hardness, gumminess, and springiness (Krasaekoopt et al., 2004;

Table 3. Chemical indexes of Kouwan Lao (KWL; mean $\pm$ SD; n = 3)

Type ¹	Protein (%)	Fat (%)	Moisture (%)	pH
H	3.28 $\pm$ 0.09 ^a	3.35 $\pm$ 0.14 ^b	84.22 $\pm$ 0.12 ^a	6.23 $\pm$ 0.21 ^a
S	3.64 $\pm$ 0.10 ^b	3.31 $\pm$ 0.02 ^b	83.19 $\pm$ 0.17 ^b	6.30 $\pm$ 0.08 ^a
P	4.72 $\pm$ 0.16 ^c	3.54 $\pm$ 0.24 ^b	79.99 $\pm$ 0.07 ^c	6.09 $\pm$ 0.13 ^a
U	4.45 $\pm$ 0.12 ^d	3.98 $\pm$ 0.04 ^a	80.22 $\pm$ 0.20 ^c	6.13 $\pm$ 0.13 ^a

^{a-d}Values with different superscripts within the same column differ significantly ($P < 0.05$).

¹H = KWL made by high temperature, long time (HTLT) milk; S = KWL made by HTLT milk with 3% skim milk powder; P = KWL made by pasteurized milk; U = KWL made by UHT milk.

Table 4. Textural indexes of Kouwan Lao (KWL) made by different types of milk; mean $\pm$ SD; n = 3

Type ¹	Hardness (g)	Springiness (mm)	Chewiness (mJ)	Cohesiveness	Gumminess (g)	Resilience (g)
H	67.9 $\pm$ 0.57 ^a	1.04 $\pm$ 0.09 ^a	25.63 $\pm$ 1.56 ^a	0.53 $\pm$ 0.01 ^a	35.60 $\pm$ 1.14 ^a	0.10 $\pm$ 0.01 ^a
S	77.3 $\pm$ 2.23 ^b	1.88 $\pm$ 0.06 ^b	28.80 $\pm$ 0.46 ^b	0.86 $\pm$ 0.04 ^b	45.57 $\pm$ 0.42 ^b	0.13 $\pm$ 0.01 ^a
P	78.8 $\pm$ 1.34 ^c	2.50 $\pm$ 0.07 ^c	32.07 $\pm$ 1.23 ^c	0.88 $\pm$ 0.03 ^b	44.50 $\pm$ 0.98 ^b	0.28 $\pm$ 0.03 ^b
U	79.5 $\pm$ 2.60 ^d	2.52 $\pm$ 0.05 ^c	32.83 $\pm$ 3.95 ^c	0.88 $\pm$ 0.07 ^b	45.03 $\pm$ 2.15 ^b	0.30 $\pm$ 0.01 ^b

^{a-d}Values with different superscripts within the same column differ significantly ($P < 0.05$).

¹H = KWL made by high temperature, long time (HTLT) milk; S = KWL made by HTLT milk with 3% skim milk powder; P = KWL made by pasteurized milk; U = KWL made by UHT milk.

Lee and Lucy, 2006; Akgun et al., 2016), and found that a more compact texture was easier to form in dairy products made by UHT milk, which was similar to our

findings. In addition, in our study, compared with H KWL sample, the S KWL samples adding 3% of skim milk powder showed a significant advantage in the tex-

A **B** **C** **D**

Figure 2. The microstructure of Kouwan Lao (KWL; A–D) made by 4 types of milk: (A) H KWL [KWL made by high temperature, long time (HTLT) milk]; (B) S KWL (KWL made by HTLT milk with 3% skim milk); (C) P KWL (KWL made by pasteurized milk); and (D) U KWL (KWL made by UHT milk).

ture feature, which was consistent with previous studies (Sandoval-Castilla et al., 2004; Akgun et al., 2016).

Effect of Milk Types on the Microstructure of KWL

The scanning electron microscopy images indicated obvious differences in microstructure of KWL samples made from different types of milk (Figure 2). Among the 4 KWL samples, the curd of the U KWL sample was the closest, smoothest, and most homogeneous; in contrast, curd quality of H KWL was the most overlooked, with large pores and rough on the curdling surface of samples. These may be because the higher preheat treatment temperature could accelerate the fusion of proteins, leading to more interconnected and dense gel networks (Lucey and Singh, 1999). In addition, compared with the H KWL sample, the microstructure of S KWL samples adding 3% of skim milk powder was smooth and uniform, which was similar to the report of Park et al. (2007), who found the microstructure of goat milk yogurt with added bovine skim milk powder was smoother and more friable. Desai et al. (2013) indicated that the addition of skim milk powder could increase the graininess, viscosity, and cohesiveness, thereby improving the microstructure of the dairy products.

CONCLUSIONS

The data of this study indicated the effects of milk types on the attributes of KWL, including microbial composition, physicochemical properties, texture properties, and microstructure. Our findings showed that the milk types significantly affected the bacterial composition rather than the fungal structure in KWL. The chemical composition of the KWL product was also related to the types of raw milk, but the pH was not affected. With the increase of heat treatment temperature and the addition of skim milk powder, the texture and microstructure of KWL were closer, smoother, and more homogeneous. Therefore, in industrial production of KWL, it is suggested to shorten the curd time and improve product quality by using UHT milk with skim milk powder.

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