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Study on *Streptococcus thermophilus* isolated from Qula and associated characteristic of acetaldehyde and diacetyl in their fermented milk

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In this study, the lactic acid bacterial population of Qula cheese from the Gansu and Sichuan provinces of China were isolated and identified. Eight strains of *Streptococcus thermophilus* were isolated, of which five strains were selected for further characterization based on their fermentation properties. The changes in a number of parameters, including titration acidity, pH, viable counts, PrtS protease activity and the production of acetaldehyde, diacetyl and organic acid, were monitored during fermentation and the storage of fermented milks produced by the respective strain. All of the strains displaying acidifying capacity and all five fermented milks maintained high viable counts of *S. thermophilus* from fermentation to storage. Our study found that the changes in the monitored parameters were strain-specific and varied considerably among the five tested strains. Fermented milks produced by strain IMAU80809 had the highest concentration of acetaldehyde and were most favorable in the sensory evaluation. This study confirms that Qula cheese is a good source for isolating novel lactic acid bacterial strains with different fermentation properties, which will be very useful for further development and industrialization of traditionally fermented dairy products.

Key Words: acetaldehyde and diacetyl; isolation and identification; Qula cheese; *Streptococcus thermophilus*

Introduction

Qula cheese is one of the home-made fermented cheese-like yak milk products. It is made from yak milk in a traditional way: defatting, acidifying, and drying in air. This kind of cheese is fermented by the interaction of distinct types of microorganisms, yeast and lactic acid bacteria (LAB). In particular, LAB plays an essential role in the formation of the aroma, texture, and acidity of the products. Previous studies showed that LAB is the dominant species in the microorganisms of Qula cheese, and play the most important role in the accumulation of nutrients (Bao et al., 2012; Duan et al., 2008). Some of the lactic acid bacteria are also known to have beneficial effects on human health (Mohd Adnan and Tan, 2007; Zhang, W. Y. et al., 2008). Because of its specific flavor, rich nutrients, and smooth texture, Qula is popular among the local consumers, which is proven by its constant presence in the local markets. In order to improve the production process and understand the role of these microorganisms in contributing to the product characteristics, it is of interest to isolate and characterize the LAB strains in Qula. Moreover, Qula may also be a useful source for isolating novel and natural LAB strains.

In recent years, with the rapid development of modern fermentation technology, a number of dairy products have been developed and industrialized based on traditional fermented dairy products. Flavor characteristics are considered as a primary target to preserve during these processes because of the consumers' acceptance and preference. This is also essential for the promotion of the dairy products (Pastink et al., 2008). Therefore, screening for

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Table 1. The sampling location and viable LAB counts on M17 agar.

Sampling location			LAB counts (log_{10} CFU g^{-1})	
Sampling province	Samples (*n*)	Sampling county (villages)*	Average	Range
Sichuan	17	Hongyuan (5)	7.18 ± 1.49	4.00–8.62
	1	Norgay (1)	8.26	
Gansu	1	Xiahe (5)	7.9 ± 0.89	5.04–9.15
	7	Luqu (5)		
	4	Maqu (2)		
	1	Cooperation City (1)		

Date represent the means (±SD) of the number of samples (*n*).
*The number of different villages sampled in each county.

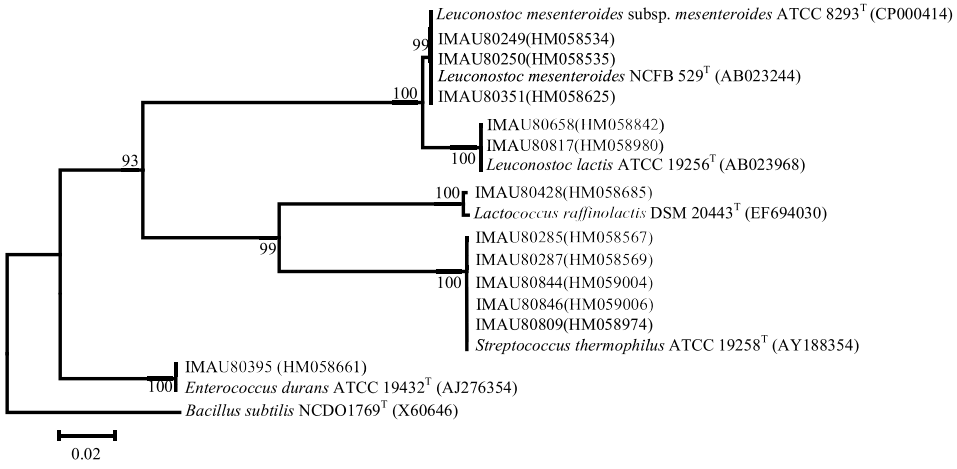

Fig. 1. Neighbor-joining tree showing the phylogenetic relationships between the isolates and the type strains based on 16S rRNA gene sequences, only some representative isolates were indicated.

Bacillus subtilis was used as the outgroup. Bootstrap values based on 100 replications are given at the nodes.

the specific strains which produce the typical flavor of the traditional dairy products is of high importance. Various volatile bacterial metabolites produced by lactic acid fermentation or other related reactions contribute to the flavor of fermented milk products (Routray and Mishra, 2011). For starter cultures, one important evaluating parameter is their aroma-producing ability, which can result from the formation of metabolites such as acetaldehyde and diacetyl (Yuguchi et al., 1989). Acetaldehyde provides the typical "yogurt-type" flavor in buttermilk and other yogurt-related products (Ott et al., 1999), while diacetyl gives the characteristic sour buttery flavor (Erkus et al., 2014).

Besides *Lactococcus lactis*, *Streptococcus thermophilus* is the second most widely used lactic acid bacterium in the industrial manufacture of fermented food products. The market value of *S. thermophilus* is estimated to be around 40 billion US dollars with a scale of over 10^{21} live cells per year for human consumption (De Vuyst and Tsakalidou, 2008; Hols et al., 2005). Rasic and Kurmann (1978) has reported the crucial role of *S. thermophilus* in producing the aromatic compounds in fermented milk products, while Beshkova et al. (1998) supported *Lactobacillus delbrückii* subsp. *bulgaricus* as the major source of production of flavor compounds. Several metabolic pathways of *S. thermophilus* have been shown to produce acetaldehyde and diacetyl in fermented milk (Chaves et al., 2002). However, due to the complex metabolic network and strain specificity, the major metabolic pathways for acetaldehyde and diacetyl are yet to be further elucidated. To our knowledge, the relationship among acetaldehyde and diacetyl production, protease activity and fermentation properties, such as bacterial viable counts and the production of organic acids, in traditionally fermented bovine milk by *S. thermophilus*, is still not fully understood.

The objectives of the present study were to isolate and identify LAB cocci in Qula and to evaluate the fermentation characteristics and flavor producing properties of the isolates. The results of the work help to identify novel starter culture strains from Qula, as well as providing reference information on how acetaldehyde and diacetyl production in the fermented bovine milk is related to other fermentation properties.

Materials and Methods

Collection of Qula samples. In this study, eighteen Qula samples were collected from widely distributed sites in the Sichuan and Gansu Provinces of China over the period 4–12, September, 2009 (Table 1). These samples were

mostly fermented using a traditional process by a local resident. Samples were collected in sterilized tubes containing $CaCO_3$ and starch, and immediately stored on ice before transportation to the laboratory for microbiological analysis.

Culture medium, cultivation and isolation methods. Samples (1 ml) were mixed in 9 ml of 0.85% (w/v) sterile physiological saline. Serial dilution (10^{-1}–10^{-8}) was prepared from the supernatant and the dilutions (10^{-5}–10^{-8}) were plated in duplicate on an M17 medium (Oxoid Ltd., Basingstoke, UK) containing 0.01% (v/v) cycloheximide and polymyxin (Bao et al., 2012). Plates were incubated under anaerobic conditions at 30°C, 37°C and 42°C for 48 h. Finally, 30°C was chosen as the best growth temperature for cocci since lower survival rates were observed with the two other considered temperatures. Colonies with distinct morphological differences, in particular the ones with irregular edge, were streaked on at least three M17 agar plates. Individual colonies were randomly picked and tested for Gram stain, cell morphology, and catalase reaction before further identification. Distinctly Gram-positive, coccoid and catalase negative isolates were grown in M17 broth at 4°C for the preparation of frozen stock in 10% (w/v) skim milk broth. Frozen stocks were stored at –80°C or lyophilized for long-term storage. In addition, all type strains were purchased from the China General Microbiological Culture Collection Center (CGMCC).

16S rRNA gene sequencing of the isolates. Cultures of the isolates were grown to late exponential growth phase before DNA extraction. The amounts of extracted DNA were quantified by UV spectrophotometer measurements. Extracted DNA was diluted to a final concentration of 100 ng μl^{-1} for further use. The 16S rRNA gene was amplified using the forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and the reverse primer 1495R (5′-CTACGGCTACCTTGTTACGA-3′) based on the method described in Sun et al. (2010). Each PCR reaction contained 100 ng DNA template, 10 pM of each primer, 200 μM of dNTPs (Takara), 50 μl PCR buffer containing 1.5 mM $MgCl_2$, 0.4 U Taq DNA polymerase (TaKaRa Corporation, Dalian, China), and the volume was adjusted to 25 μl with ultrapure water. The PCR amplifying procedure was as follows: 5 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 58°C, 2 min at 72°C and then 10 min at 72°C; it was carried out on an automatic thermal cycler (MJ Re-search PTC-200). PCR products (~1,500 bp fragments) were electrophoresed in a 1.0% agarose gel and visualized by UV transillumination after ethidium bromide staining.

Positive PCR products were sent to Shanghai Sangni Biosciences Corporation of China for DNA sequencing. The 16S rRNA gene sequences of all isolates were submitted to the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov) for BLAST search. Sequences were imported into MEGA version 4.0 software (Tamura et al., 2007), with which a sequence alignment and phylogenetic tree were created on the basis of the neighbor-joining method.

Proteinase assay. The PrtS proteinase phenotype of the isolates was determined by Fast Slow Difference Agar medium after 48 h cultivation at 42°C, according to Huggins and Sandie (1984). Strains LMD-9 ($PrtS^+$, from ATCC) and ND03 ($PrtS^-$, from LABCC) were used as controls.

Milk fermentation. Whole milk powders (Fonterra Ltd., Auckland New Zealand) contained 39.1% lactose and 25.0% proteins (with a casein content above 34%) were used in this study. The whole milk powders were hydrated (11.5%, w/w) and supplemented with 6.5% sucrose (w/w). The hydrated milks were heated to 85°C for 30 min, cooled to 4°C and inoculated with the isolates at an inoculation level of 5×10^6 CFU g^{-1}. After inoculation, the milks were incubated at 37°C. Samples were taken every two hours for the determination of the pH value, titratable acidity (TA), and viable count until the pH value reached 4.5.

Fermentation characteristics. The pH values were measured using a pHFE20 pH meter (Shanghai, China). The titratable acid (TA) was determined by mixing a 5 g sample with 40 ml of sterile distilled water and titrated with 0.1 N NaOH using 0.5% phenolphthalein as indicator. The viable counts of *S. thermophilus* were enumerated on M17 agar according to the description of Tharmaraj and Shah (2003).

The fermentation time (time required for the samples to reach pH 4.5) for each strain was recorded. Fermented milks were then stored at 4°C, and samples were taken every 12 hours. The pH value, titratable acidity (TA), and viable count were monitored during the sample storage. Meanwhile, the content of acetaldehyde was also monitored by an acetaldehyde kit, K-ACHYD (Megazyme, Ireland), based on the enzymatic (acetaldehyde dehydrogenase) reduction of NAD to $NADH^+$ (Chaves et al., 2002). Furthermore, diacetyl was quantitatively determined by dynamic headspace analysis using the procedures of Beshkova et al. (1998). Briefly, a 1 g sample was mixed with 3 ml of 1 M HCL in a 10 ml centrifuge tube before centrifugation at 4000 g for 10 min. One milliliter of supernatant was collected for determining the organic acid, filtered through a 0.45 μm membrane and stored at –80°C until further analysis by HPLC.

HPLC analysis of organic acids. The concentrations of lactic and formic acids in samples were determined using HPLC (Agilent 1100; Agilent Technologies Inc., Santa Clara, CA), according to the method of Zhang, H. et al. (2008). Samples were prepared as described previously. The filtrates were allowed to pass through an Agilent Zorbax SB C18 column maintained at 35°C. The degassed mobile phase was 10 mM phosphate buffer (pH 2.5)/methanol (v/v, 97/3), which was used at a flow rate of 0.5 ml min^{-1}. Signals were monitored with a multi-wavelength fluorescence detector set at 210 nm.

Sensory evaluation. Descriptive sensory analysis was performed by a panel of ten trained panelists at 0, 12, 24, 36 and 48 h after storage of fermented milks. Panel members discussed and agreed upon the definitions and how to score the attributes on the scale. The panelists and test rooms of the sensory analysis satisfied the international standards (ISO-8586-1 1993) and (ISO-8589 1988), respectively. 100-ml beakers filled with set-type fermented milks were used for evaluation. Tablespoons and water were used to rinse out the testers' mouths between con-

secutive samples. The evaluated sensory characteristics included appearance (firmness, shape-maintenance and surface-smoothness), texture (hardness, consistency and flatness), flavor (acid and global flavor) and taste (acid taste, bitter taste, astringency, greasy texture and global taste) (Buono et al., 1990; Macedo et al., 1999; Salvador and Fiszman, 2004). Each sub-item was divided into five grades (five scores: 1 = strongly dislike, 2 = dislike, 3 = common, 4 = like, 5 = strongly like). Item and total scores were calculated and analyzed by SPSS software.

Results and Discussion

Enumeration, isolation and identification of LAB from Qula cheese

Most of the isolates were considered as presumptive LAB cocci characterized by their positive Gram reaction, the absence of catalase, and cell morphology. The viable counts of cocci and the sampling location are shown in Table 1. Total counts of the cocci in M17 agar varied from 4.00 to 9.15 $\log_{10}$ CFU g^{-1}. The different ripening stages of the collected Qula samples, the way of sample transportation, and the sampling region, might affect together to the variation of bacterial counts.

All the isolates were identified to species level by 16S rRNA gene sequencing, showing a greater than 99% similarity to the reference strains. A total of 24 isolates and their related type strains were chosen to construct a phylogenetic tree by the MEGA software (Fig. 1). As shown in Fig. 1, all representative strains are grouped together with the corresponding type strains. Based on the 16S rRNA gene sequences analysis, 24 isolates were classified to 6 species: *Enterococcus durans* (IMAU80395), *Lactococcus raffinolactis* (IMAU80428), *Leuconostoc lactis* (IMAU80817, IMAU80818, IMAU80408, IMAU80656, IMAU80657 and IMAU80658), *Leuconostoc mesenteroides* (IMAU80248, IMAU80249, IMAU80250, IMAU80251, IMAU80350 and IMAU80351), *Leuconostoc mesenteroides* subsp. *mesenteroides* (IMAU80286 and IMAU80305) and *Streptococcus thermophilus* (IMAU80806, IMAU80809, IMAU80285, IMAU80287, IMAU80844, IMAU80845, IMAU80846 and IMAU80847). Among the identified taxa, both *Leuconostoc* and *S. thermophilus* were widely found. However, *S. thermophilus* dominated, contributing to 33% of all cocci isolates. Bao et al. (2012) also reported that *S. thermophilus* was the dominating species in Qula cheese (39%), whereas Tan et al. (2010) and Duan et al. (2008) reported that the dominating LAB was the *Leuconostoc* species (86% and 78%, respectively).

Fermentation characteristics

Among the isolated *S. thermophilus* strains, we selected five isolates, IMAU80844, IMAU80846, IMAU80285, IMAU80287 and IMAU808029, for further characterization. The changes in viable counts, pH value, and TA, during milk fermentation with these five isolates are shown in Fig. 2. An obvious increase in TA and decrease in pH in milk were observed with all of the strains during the fermentation period. Due to the rapid acidification ability of *S. thermophilus*, it took less than 6 h for the milk samples

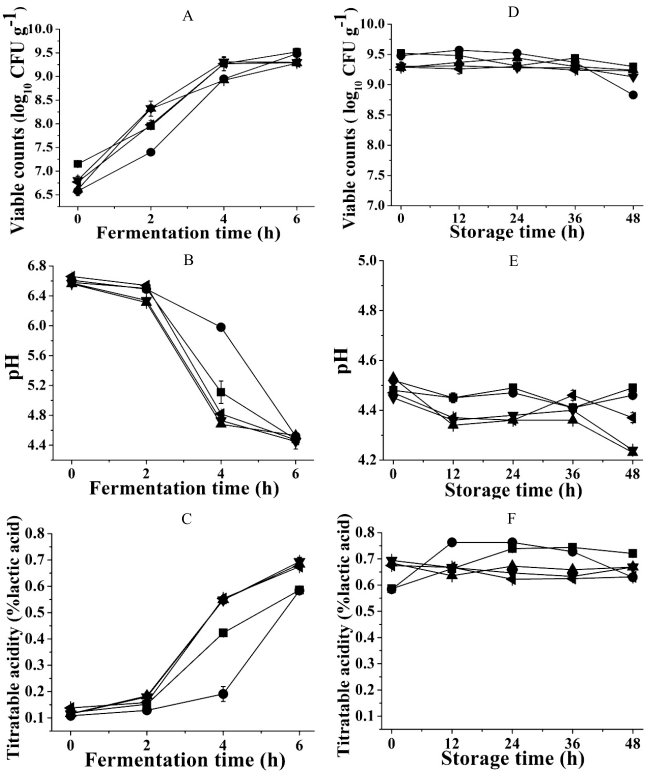

Fig. 2. Changes in fermented properties (viable counts, A and D; pH, B and E; TA, C and F) during milk fermentation and storage by five *S. thermophilus* strains.

Black squares = *S. thermophilus* IMAU80844; black circles = *S. thermophilus* IMAU80846; black upper triangles = *S. thermophilus* IMAU80285; black lower triangles = *S. thermophilus* IMAU80287; black oblique triangles = *S. thermophilus* IMAU80809. Data are shown as the mean ± S.E.M., $n = 3$.

to reach a pH value of around 4.5. IMAU80285 showed the shortest fermentation time of 4.48 h and IMAU80846 was the slowest strain with a fermentation time of 6 h. Three strains (IMAU80285, IMAU80287 and IMAU80809) were considered as fast acidifying candidates for yogurt starter culture which could reduce the pH to 4.6 within 5 hours (Erkus et al., 2014). At the end of the fermentation, an average TA of around 0.6% lactic acid was recorded with all the strains. IMAU80287 had the highest value of TA of about 0.69% lactic acid, while the other two strains (IMAU80844 and IMAU80846) showed a relatively low ability of acidification. All five strains were above 0.6% acidity within 6 h fermentation and can be considered as fast acid-producing strains for yogurt production according to Raquib et al. (2003). Soomro and Masud (2008) found that dahi yogurt derived *S. thermophilus* were mostly fast acidifiers, while all *S. thermophilus* from fermented milk laban showed low acid-producing capabilities (Chammas et al., 2006), suggesting that *S. thermophilus* from different sources of dairy products exhibited various acidifying capabilities.

A high density of viable *S. thermophilus* in the fermented milk reflects a high proteolytic activity of the strains (Letort and Juillard, 2001), which is demonstrated by the cheese derived *S. thermophilus*. Most of them exhibit high proteolytic activity (Erkus et al., 2014). In this study, the

average viable counts of the isolates reached a value of over 9.20 $\log_{10}$ CFU g^{-1} at the end of the fermentation process. Among the 5 tested strains, the strain IMAU80844 gave the highest viable counts of 9.59 $\log_{10}$ CFU g^{-1} in fermented milk. These results together demonstrate that the *S. thermophilus* strains isolated in the current study possess desirable fermentation properties of starter cultures.

Protease activity in S. thermophilus

PrtS is a key enzyme of the proteolytic activity, initiating the breakdown of caseins into oligopeptides before their uptake, and PrtS gene is harbored on the chromosome in *S. thermophilus* (Delorme et al., 2010). Only IMAU80846 showed the positive phenotype and the others were $PrtS^-$ strains (data not shown). Shahbal et al. (1991) reported that three of 97 *S. thermophilus* strains displaying the highest proteolytic activities ($PrtS^+$) also showed the highest acidifying capacities. Galia et al. (2009) also found that among a collection of 30 *S. thermophilus* strains, 15 strains had displayed PrtS activities and reported that the strains displaying the highest acidifying capacities were all $PrtS^+$. There is an obvious distinction between our result and the above-mentioned views, which may reflect the difference in the isolation sources used. In fermented milk, flavor compounds such as acetaldehyde and diacetyl, could be basically formed from glucose and threonine metabolism (Bratovanova, 1999; Ott et al., 2000b). Smit et al. (2005) reported that amino acids resulting from proteolysis are the major precursors of specific flavor compounds, such as aldehydes, acids and esters. In this study, the $PrtS^+$ strain IMAU80846 had a considerable acidifying capacity (Fig. 2F) and high viable counts (Fig. 2D) during storage, which is consistent with previous research (Fernandez-Espla et al., 2000) and may contribute to the flavor development such as the production of acetaldehyde and diacetyl.

Changes in pH, TA and viability of S. thermophilus during storage

The changes in pH value and TA during storage of the fermented milk samples were shown in Figs. 2E and 2F. During the 36–48 h of storage, the pH of fermented milks produced by IMAU80285, IMAU80287 and IMAU80809 decreased with minor changes in TA. In contrast, fermented milks produced by IMAU80844 and IMAU80846 had the opposite trend. Purwandari (2009) reported that the storage time and differences between strains significantly affected TA. She also observed that two strains of *S. thermophilus* had a distinct acid-producing period during storage. Post-acidification is one of main problems for yogurt sensory quality during shelf life (Donkor et al., 2006). Owing to the TA stability and mild post-acidification, IMAU80285, IMAU80287 and IMAU80809 are preferential over the other strains for yogurt production. No dramatic change was observed in the viable count of *S. thermophilus* during fermented milk storage (Fig. 2D) except for the strain IMAU80846. Generally, slight decreases in the number of viable bacteria were observed. It is well established that *S. thermophilus* growth was restricted due to a low tolerance to a pH of lower than 5.5

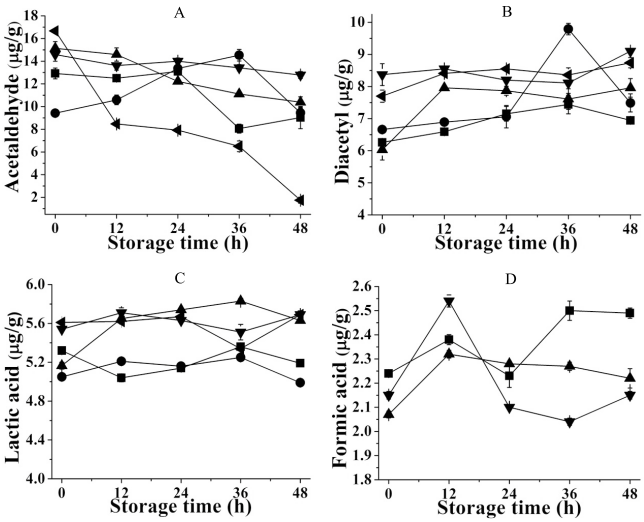

Fig. 3. Changes in the content of acetaldehyde (A), diacetyl (B), lactic acid (C) and formic acid (D) during storage of fermented milks produced by five *S. thermophilus* strains.

Black squares = *S. thermophilus* IMAU80844; black circles = *S. thermophilus* IMAU80846; black upper triangles = *S. thermophilus* IMAU80285; black lower triangles = *S. thermophilus* IMAU80287; black oblique triangles = *S. thermophilus* IMAU80809. Formic acid is not found in detectable levels with IMAU80846 and IMAU80809. Data are shown as the mean ± S.E.M., $n = 3$.

(Pinto et al., 2009). The results were in agreement with the earlier study by Birollo et al. (2000).

Changes in acetaldehyde, diacetyl, lactic acid and formic acid of fermented milk during storage

In this study, the changes in acetaldehyde, diacetyl, lactic acid and formic acid during the storage of fermented milks are monitored (Fig. 3). Generally, the production of these compounds is affected by strain-specific traits. Acetaldehyde is a major flavor compound in fermented dairy products, as reviewed by Hamdan et al. (1971). It was observed that IMAU80285 had the highest content of acetaldehyde at first, but it declined upon storage. In contrast, IMAU80846 showed a gradual increase in acetaldehyde during storage of 12–36 h. The breakdown of acetaldehyde during storage possibly resulted from the microbial enzymes driven-hydrolysis and may provide amino acids and nucleotides for maintaining the cell viability (Güler-akn, 2005). Similarly, Xanthopoulos et al. (2001) observed the reduction of acetaldehyde in some *S. thermophilus* strains during fermented milk storage. Moreover, diacetyl, mainly produced by *S. thermophilus*, is another antibacterial compound potentially contributing to the yogurt aroma (Chammas et al., 2006; Kang and Fung, 1999; Routray and Mishra, 2011). In our study, IMAU80846 produced the highest content of diacetyl of 9.79 $\mu g\ g^{-1}$ after storing for 36 h along with a decrease of the viable counts. This contrary trend above might mean that diacetyl formation and the proliferation of bacteria competed for nutrients such as free amino acids. In addition, Beshkova et al. (1998) reported that *S. thermophilus* strains produced a higher acetaldehyde amount than diacetyl content which was consistent with our data. Many other compounds were also found to contribute to the

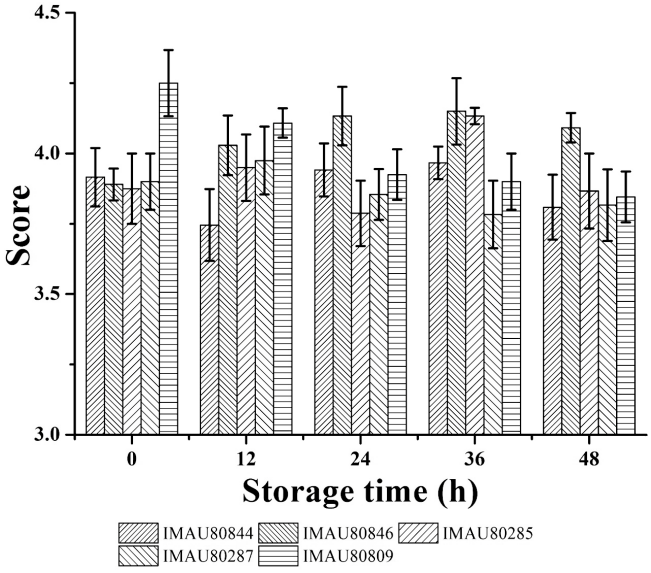

Fig. 4. Sensory evaluation of yogurts produced by five *S. thermophilus* strains after different storage times.

Data are shown as the mean ± S.E.M., $n = 10$.

aroma of fermented milk, including lactic acid and formic acid (Kleerebezem et al., 2000). Lactic acid and formic acid could be detected as distinct peaks in our HPLC analysis with retention times of 4.3 and 4.9 min, respectively. The main flavoring agent in yogurt is lactic acid, which contributes to its sour and refreshing taste (Ott et al., 1997). There is no significant correlation between lactic acid content and strain viable counts in our study. Xanthopoulos et al. (2001) reported that the acidification ability of *S. thermophilus* of 74 strains showed no obvious relation to the final amount of lactic acid produced. Formic acid is also an important metabolite of lactic acid fermentation and its production is strain-dependent (Perez et al., 1991). Formic acid was found in detectable levels in three of the studied strains (IMAU80287, IMAU80846 and IMAU80809) during storage, but not with the others (IMAU80846 and IMAU80809). IMAU80287 had the highest ability to produce formic acid, with a peak at 12 h of storage (Fig. 3D). It is suggested that formic acid-producing strains could convert to heterolactic (or mixed-acid) fermentation under certain conditions as reported by Mehmeti et al. (2011).

In addition, the diverse physiological and biochemical properties represent the degree of phenotypic diversity existing within the species. Thus, the role of *S. thermophilus* in the fermentation of milk is not related only to the production of lactic acid, it also has several other important properties, such as sugar metabolism, galactose utilization and proteolytic activity according to Iyer et al. (2010).

Sensory evaluation of yogurts

The sensory evaluation of taste was taken at different time points after storage (0, 12, 24 and 36, 48 h) (Fig. 4). The overall scores of IMAU80846 and IMAU80809 were better than the other 3 strains; however, IMAU80809 had the most acceptable score at the beginning of storage while the content of acetaldehyde showed the same trends. Based on our results, the sensory quality decreased with duration and was found to be closely related to changes in the content of aromatic compounds, especially acetaldehyde and diacetyl. By comparing the results of IMAU80287 and IMAU80844, it was found that the important flavor differences between these two samples were mainly due to acidity but not aroma compounds. Similar results were also reported by Ott et al. (2000a).

Conclusions

The objective of this study was to analyze the aroma compounds produced by *S. thermophilus* strains isolated from a traditional dairy product, Qula cheese, in China. Eight strains of *S. thermophilus* were isolated from Qula cheese and they were the dominant LAB population within the samples. Among them, 5 strains were chosen for further characterization. Parameters, including TA, pH, viable counts, protease and production of acetaldehyde, diacetyl and organic acid, were monitored. Based on our results, these monitored parameters were strain-specific. Fermented milks produced by IMAU80809 had the highest concentration of acetaldehyde and were the most favorable in the sensory evaluation. This study confirms that Qula cheese is a good source for isolating novel LAB strains of different fermentation properties, which will be very useful for further development and industrialization of traditionally fermented dairy products. The strains isolated in this study are valuable resources deserving future detailed characterization.

Acknowledgments

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