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Identification and characterization of lactic acid bacteria isolated from Tibetan Qula cheese

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Fourteen strains of Lactic Acid Bacteria (LAB) isolated from Qula, a Tibetan traditional yak cheese, were divided into four groups (A–D) according to morphological and biochemical characteristics. On the basis of the 16S rRNA gene sequence analysis, group A and group B strains were placed in the cluster making up the genus *Leuconostoc*, which together with *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides*, formed a distinct cluster. The group C strain was clearly identified as *Enterococcus faecium* by forming a very well defined cluster with this species. The group D strains were placed in the lactobacilli cluster with *Lactobacillus plantarum* and *Lactobacillus pentosus* being the closely related species. On the basis of DNA-DNA hybridization, strains in the groups A, B, C and D were identified as *Leuconostoc mesenteroides* subsp. *dextranicum*, *Leuconostoc pseudomesenteroides*, *Enterococcus faecium* and *Lactobacillus plantarum*, respectively. *Leuconostoc mesenteroides* subsp. *dextranicum* was the dominate member of the population.

Key Words—DNA-DNA hybridization; lactic acid bacteria; Qula cheese; 16S rRNA gene sequence; yak milk

Introduction

Qula is a grainy, hard, yellow or white cheese made in the Tibetan plateau from yak's milk. It has been a traditional food of the Tibetans for more than 1,000 years. Qula always made in the summer time by adding yoghurt to yak's milk. It is an important method of storing food protein and is used as a starter for making yoghurt.

The Tibetan plateau is an area in northwest China and other countries with an average altitude of over 4,000 m (Wu, 2001). It is a distinct ecological region

with a wide biodiversity. Such diversity of species has an impact on the formation of the specific microflora found in yak's milk and in Qula (Li et al., 2002; Luo et al., 2005).

Many kinds of cheese made from unpasteurized milk through traditional manufacturing procedures may possess a very diverse and rich microflora (Centeno et al., 1996; Flórez et al., 2006; Medina et al., 2001; Mustafa, 2006; Prodromou et al., 2001; Terzic-Vidojevic et al., 2006). Some researchers have reported that the local microflora of raw milk influences the biochemical characteristics of cheese (Demarigny et al., 1997; Marino et al., 2003; Prodromou et al., 2001). The microflora play a fundamental role in the fermentation of Qula; the biodiversity of bacteria involved in cheese production can be considered a basic factor in the formation of the typical features of traditional Qula prod-

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ucts. Since Qula is kept more than 2–3 years at room temperature in local areas it contains different microorganisms and abundant lactic acid bacteria.

In the present study, the LAB strains isolated from six samples of Qula were characterized, taking particular interest in species that are likeliest to play an important role in the Qula fermentation process. To determine their taxonomic status, the isolates were also studied by 16S rRNA gene sequence analysis and DNA-DNA hybridization experiments.

Materials and Methods

Qula samples. Five Qula samples were collected from Qinghai province and one from Gansu province, Tibetan plateau pasturing areas of China (Table 1). These Qula were made on local yak farms using the process shown in Fig. 1. The samples were collected in sterilized bags, kept in ice boxes and transported to the microbiology laboratory (Zhengzhou University, Henan, China) for analysis. Upon their arrival at the laboratory, they were analyzed immediately or kept at 4°C for as short a time as possible before analysis.

Bacterial isolates. Qula samples (5 g) were blended with 45 ml sterilized water, and were 10^{-1} to 10^{-5} serially diluted in sterilized water. The numbers of LAB were measured by the plate count on MRS agar (Difco Laboratories, Detroit, USA) incubated at 30°C for 48 h under anaerobic conditions. Coliform bacteria were counted on blue light broth agar (Nissui Ltd., Tokyo, Japan) incubated at 30°C for 48 h; mold and yeast were counted on Potato Dextrose agar (Nissui) incubated for 24 h at 30°C. Yeast were distinguished from mold or bacteria by colony appearance and observation of cell morphology. Bacilli and aerobic bacteria were distinguished by the colony shape and counted on Nutrient agar (Nissui) incubated for 24 h at 30°C under aerobic conditions.

Each LAB colony was purified twice by streaking on

MRS agar. The purified strains were stored at -80°C with nutrient broth (Difco) and dimethyl sulfoxide in a ratio of 9:1 for further analysis (Cai et al., 1998b; 1999b). Colonies were counted as viable numbers of microorganisms (colony forming unit [CFU] g^{-1} of fresh matter [FM]).

Morphological, physiological, and biochemical tests. Gram stain of LAB and morphological characteristics were determined after 24 h of incubation on MRS agar. Catalase activity and gas production from glucose were determined as described (Kozaki et al., 1992). Growth at different temperatures was detected in MRS broth after incubation at 10 and 15°C for 10 d, and at 45°C and 50°C for 7 d. Growth at pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.5, 8.0, and 9.0 was observed in MRS broth after incubation at 30°C for 7 d (Cai et al., 1999d). Salt tolerance of LAB was tested in MRS broth containing 3.0% and 6.5% NaCl. Carbohydrate assimilation and fermentation of 49 different compounds with one control were identified on API 50CH strips (Cai et al., 1999a, Ennahar et al., 2003).

16S rRNA gene sequence analysis. Cells grown for 8 h in MRS broth at 30°C were used for DNA extraction and purification. The 16S rRNA sequence coding region was amplified by polymerase chain reaction (PCR) and performed in a PCR ThermalCycler (GenAmp PCR System 9700; PE Applied Biosystems, Foster City, California, USA) and reagents from a Takara Taq PCR Kit (Takara Shuzo Co., Ltd., Otsu, Japan) (Cai et al., 1999c). Sequencing was performed twice on both strands by the dideoxy method using a PRISM BigDye Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems) in combination with an Applied Biosystems model 310A automated sequencing system.

Searching 16S rRNA gene sequence similarity was performed at GenBank data library by using the BLAST program. Then the sequence information was introduced into the CLUSTAL W software program

Table 1. Collection of Qula cheese and strains used in this study.

Sample	Collection place	Strain
Qula A	Taer-temple Yak Farm, Huangzhong, Qinghai	QL2, QL28
Qula B	Shizuishan Yak Farm, Shizuishan, Qinghai	QL5, QL29
Qula C	Zhangye Yak Farm, Zhangye, Gansu	QL30
Qula D	Hatu Yak Farm, Qinghai Lake, Qinghai	QL6, QL31, QL32, QL33, QL34
Qula E	Tianjun Yak Farm, Tianjun, Qinghai	QL8, QL35
Qula F	Baya Yak Farm, Datong, Qinghai	QL10, QL11, QL36

Fig. 1. The process of making Qula cheese by traditional Tibetan methods.

(Hitachi Software Engineering Co.) for assembly and alignment. The 16S rRNA gene sequences of isolates were compared with sequences from the type strains of LAB held in the GenBank. Nucleotide substitution rates were calculated, and phylogenetic trees were constructed by the neighbor-joining method. *Bacillus subtilis* NCDO 1769 was used as an outgroup organism (Cai et al., 1998a, c, 1999c). The topologies of trees were evaluated by bootstrap analysis of the sequence data with CLUSTAL W software based on 100 random resamplings (Thompson et al., 1994).

Nucleotide sequence accession number. The nucleotide sequences for the 16S rRNA gene described in this report were deposited with GenBank under accession no. AB326298, AB326299, AB326300, and AB326301 for the representative strains QL 2, QL 28, QL 30, and QL 31 from groups A, B, C, and D, respectively.

DNA base composition and DNA-DNA hybridization. DNA extraction and purification was performed as described by Saitou and Miura (1963). DNA basic composition was determined by the method of Tamaoka

and Komagata (1984) using high-performance liquid chromatography by enzymatic digestion of DNA to deoxyribonucleosides. The equimolar mixture of four deoxyribonucleotides in a Yamasa GC Kit (Yamasa Shoyu Co., Ltd., Choshi, Japan) was used as the quantitative standard. The DNA-DNA relatedness was determined by the method using photobiotin and microplates described by Cai (Cai et al., 1999b).

Results

Counts of microorganisms

The counts of viable microorganisms in Qula samples are shown in Table 2. Overall 10^3 to 10^7 (CFU g^{-1} of FM) LAB, 0 to 10^3 coliform bacteria, 10^4 to 10^8 aerobic bacteria, 0 to 10^5 mold, and 10^3 to 10^8 yeast, 0– 10^5 bacilli were found in the Qula samples.

Morphological, physiological, and biochemical properties

The characteristics of LAB are shown in Tables 3 and 4. All isolates were Gram-positive and catalase-

Table 2. Micorbiological analysis of the Qula cheese.

Sample	Counts (cfu g ⁻¹ of FM) of viable microorganisms ^a					
	Lactic acid bacteria	Bacillus	Coliform bacteria	Aerobic bacteria	Mold	Yeast
Qula A	4.5×10 ⁴	ND ^b	ND	1.4×10 ⁴	ND	5.0×10 ⁴
Qula B	5.0×10 ⁴	5.5×10 ⁵	4.5×10 ³	1.1×10 ⁸	1.0×10 ⁵	1.3×10 ⁸
Qula C	3.0×10 ³	5.5×10 ⁵	ND	9.0×10 ⁵	5.0×10 ³	8.5×10 ³
Qula D	4.5×10 ⁷	2.4×10 ⁴	2.5×10 ³	3.2×10 ⁶	ND	3.0×10 ⁶
Qula E	2.5×10 ⁶	6.5×10 ³	ND	2.0×10 ⁵	5.0×10 ²	6.5×10 ⁵
Qula F	1.2×10 ⁴	ND	ND	2.0×10 ⁴	5.0×10 ²	1.4×10 ⁴

^a CFU, colony forming unit; FM, fresh matter.^b ND, not detected.

Table 3. Characteristics of lactic acid bacteria strains isolated from Qula cheese.*

Characteristics	Group A										Group B	Group C	Group D	
	QL 2	QL 5	QL 6	QL 8	QL 10	QL 11	QL 29	QL 32	QL 34	QL 36	QL 28	QL 30	QL 31	QL 35
Shape	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Rod	Rod
Gas from glucose	+	+	+	+	+	+	+	+	+	+	+	—	—	—
Fermentation type	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Homo	Homo	Homo
Optical form of lactate	D(—)	D(—)	D(—)	D(—)	D(—)	D(—)	D(—)	D(—)	D(—)	D(—)	D(—)	L(+)	DL	DL
Growth at temperature (°C):														
10	+	+	+	w	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+
45	+	+	w	w	+	—	+	w	w	w	—	+	+	+
50	w	w	w	w	+	—	—	—	—	—	—	+	+	+
Growth in NaCl:														
3.00%	+	+	+	+	+	+	+	+	+	w	+	+	+	+
6.50%	+	+	+	+	+	+	w	+	+	w	w	+	+	+
Growth at pH:														
3	+	+	w	+	+	+	w	+	+	+	+	w	+	+
3.5	+	+	w	+	+	+	w	+	+	+	+	w	+	+
4	+	+	w	+	+	+	w	+	+	+	+	w	+	+
4.5	+	+	w	+	+	+	w	+	+	+	+	w	+	+
5	+	+	+	+	+	+	w	+	+	+	+	w	+	+
5.5	+	+	+	+	+	+	w	+	+	+	+	w	+	+
6.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	w	+	+	w	+	w	+	+	w	w	+	+	+	+
9	w	+	w	w	w	w	+	+	w	w	+	+	+	+

* All strains were gram-positive, catalase negative. +, Positive; —, negative; w, weakly positive; Homo, homofermentative; Hetero, heterofermentative.

Table 4. API 50 CH fermentation patterns of lactic acid bacteria strains isolated from Qula cheese*

Carbohydrate	Group A										Group B	Group C	Group D	
	QL 2	QL 5	QL 6	QL 8	QL 10	QL 11	QL 29	QL 32	QL 34	QL 36	QL 28	QL 30	QL 31	QL 35
Ribose	+	+	—	—	+	—	w	+	—	—	+	+	+	+
D-Xylose	+	+	—	+	—	—	+	—	—	+	—	—	—	—
Galactose	—	—	—	—	—	—	+	—	—	—	+	+	+	+
Glucose	+	+	+	+	+	+	+	w	w	+	+	+	+	+
Fructose	+	+	+	+	+	+	w	+	+	—	+	+	+	+
Mannose	+	+	—	+	—	—	—	—	w	—	+	+	+	+
Mannitol	+	+	+	+	+	+	—	—	—	—	+	—	+	+
α -Methyl-D-mannoside	—	—	—	—	—	—	—	—	—	—	—	—	—	—
α -Methyl-D-glucoside	+	+	—	—	+	+	w	—	+	+	—	—	—	—
N-Acetyl-glucosamine	+	+	+	+	+	+	w	—	—	—	+	+	+	+
Amygdalin	—	—	—	—	—	—	—	—	—	—	+	—	+	+
Arbutin	—	—	—	—	—	—	—	—	—	—	—	+	+	—
Esculin	+	—	+	+	w	w	+	—	—	—	—	+	+	—
Salicin	—	—	w	+	—	—	w	—	—	—	—	+	+	—
Cellobiose	—	—	—	+	—	—	—	—	—	—	—	+	+	*
Maltose	+	+	—	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	—	+	+	+	+	+	+
Melibiose	—	+	—	—	—	—	+	—	—	—	—	+	—	—
Sucrose	+	+	—	+	—	—	+	—	+	+	+	+	+	+
Trehalose	+	+	—	+	—	—	+	+	—	+	—	w	—	—
Melezitose	—	—	—	—	—	—	—	—	—	—	+	—	+	+
Raffinose	—	+	—	—	—	—	+	w	—	—	—	—	—	—
Gentiobiose	—	—	—	+	—	—	—	—	—	—	+	w	w	w
D-Turanose	+	—	—	—	+	+	—	—	—	+	—	—	+	—
D-Tagatose	—	—	—	+	—	—	—	—	—	—	—	—	—	—
D-Arabitol	—	—	—	—	—	—	—	—	—	—	w	—	w	w
L-Arabitol	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Gluconate	w	—	—	+	—	—	—	—	—	—	w	—	w	w
2-K-gluconate	w	w	—	—	—	—	w	—	w	w	—	—	—	—
5-K-gluconate	—	—	—	—	—	—	—	—	w	w	—	—	—	—

* All strains gave negative results for glycerol, erythritol, D-arabinose, L-arabinose, L-xylose, adonitol, sorbose, rhamnose, dulcitol, inositol, sorbitol, inulin, starch, glycogen, xylitol, D-fucose, D-lyxose, L-fucose and L-arabitol. +, positive; —, negative; w, weakly positive.

negative bacteria. All of them were able to grow at pH values from 3.0–9.0, at 10°C and 15°C, and in 6.5% NaCl. Strains QL 11 and QL 28 were unable to grow at 45°C, and strains QL 29, QL 32, QL 34 and QL 36 not above 50°C. According to the morphological, physiological, and biochemical properties, those strains were divided into four groups, each one displaying distinct characteristics (Table 3). Strains in all groups pro-

duced acid from glucose. Group A, which included strains QL 2, QL 5, QL 6, QL 8, QL 10, QL 11, QL 29, QL 32, QL 34 and QL 36, was heterofermentative cocci that produced gas from glucose, formed D(–) isomer of lactate, and did not produce acid from amygdalin or melezitose. The phenotypic characteristics of group B were similar to group A in that its strains produced gas from glucose and formed D(–) isomer of

lactate. This group included a single strain, QL 28, that produced acid from amygdalin and melezitose, which made it different from group A. Group C, which included strain, QL 30, was homofermentative cocci and produced lactate as isomers of L(+). Group D, which included strains QL 31 and QL 35, was homofermentative rods and produced lactate as isomers of DL, and was easily distinguished from groups A, B and C (Table 4).

Molecular classification of LAB

Phylogenetic trees are shown in Fig. 2. Following phylogenetic analysis, groups A and B strains were placed in the cluster making up the genus *Leuconostoc*, which both formed a distinct cluster together with *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides*, supported with a bootstrap

value of 100%. Strains in group A were clearly assigned to the *L. mesenteroides*, since it grouped on the phylogenetic tree together with *L. mesenteroides* subsp. *dextranicum*, *L. mesenteroides* subsp. *cremoris* and *L. mesenteroides* subsp. *mesenteroides* in the 100% bootstrap cluster. Furthermore, these strains appeared to be equally linked with 3 subspecies of *L. mesenteroides* and their 16S rRNA gene sequence showed a similarity of over 99.6% to 3 subspecies of *L. mesenteroides*. Group C strain QL 30 was placed in the enterococci cluster, since this strain grouped on the phylogenetic tree with *Enterococcus faecium*, *Enterococcus durans* and *Enterococcus mundtii*. Group D strains QL 31 and QL 35 were placed in the lactobacilli cluster, since they grouped on the phylogenetic tree with *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus paraplantarum* and *Lactobacillus arizo-*

Fig. 2. Phylogenetic tree showing the relative position of *Enterococcus* (*E.*), *Lactobacillus* (*L.*) and *Leuconostoc* (*Le.*) species as inferred by the neighbour-joining method with 16S rRNA gene sequences.

Bootstrap values for a total of 100 replicates are shown at the nodes of the tree. References of the type strains used for comparison are given, as well as the GenBank/EMBL/DBJ accession number for all 16S rRNA gene sequence (in parentheses). *Bacillus subtilis* was used as an outgroup. Bar, 1% sequence divergence.

Table 5. DNA base composition and levels of DNA-DNA hybridization for *Leuconostoc*, *Enterococcus* and *Lactobacillus* species.

Strain ^a	G+C content (mol%)	DNA-DNA reassociation (%) with:									
		Group A	Group B	JCM 9700 ^T	JCM 6124 ^T	JCM 9696 ^T	Group C	JCM 5803 ^T	JCM 5804 ^T	Group D	JCM 1149 ^T
Group A (QL 2)	39.0	100	16.8	95.6	54.3	20.6					
Group B (QL 28)	38.2	15.6	100	18.3	12.7	90.3					
<i>Leuconostoc mesenteroides</i> subsp. <i>dextranicum</i> JCM 9700 ^T	39.3	98.7	14.2	100	55.3	18.0					
<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> JCM 6124 ^T	38.8	58.2	17.5	58.1	100	15.2					
<i>Leuconostoc pseudomesenteroides</i> JCM 9696 ^T	38.9	20.3	88.3	17.0	15.4	100					
Group C (QL 30)	38.4						100	13.2	92.1		
<i>Enterococcus faecalis</i> JCM 5803 ^T	39.7						9.6	100	13.7		
<i>Enterococcus faecium</i> JCM 5804 ^T	38.1						95.8	15.4	100		
Group D (QL 31)	44.9									100	90.2
<i>Lactobacillus plantarum</i> JCM 1149 ^T	45.2									84.2	100
<i>Lactobacillus pentosus</i> JCM 1558 ^T	44.1									17.8	22.3

^a Group A included QL 2, QL 5, QL 6, QL8, QL 10, QL 11, QL 29, QL 32, QL 34 and QL 36; group D included QL 31 and QL 35. Strains in groups A and D exhibited intragroup DNA homology values of over 92.5% and 95.6%.

nensis with a 100% bootstrap level. *L. plantarum* and *L. pentosus* were the most closely related species and their 16S rRNA gene sequence showed a similarity of 99.6% to *L. plantarum* and 99.5% to *L. pentosus*.

DNA base composition and DNA-DNA hybridization

The DNA base composition and DNA-DNA hybridization are shown in Table 5. Strains in groups A and B had a guanosine and cytosine content range of 38.2 to 39.0 mol%. Strains in groups C and D had 38.4 and 44.9 mol% of G+C content. The data for groups A, B, C and D were within the range of G+C content for the genus *Leuconostoc*, *Enterococcus* and *Lactobacillus*.

Strains in groups A and D exhibited intragroup DNA homology values of over 92.5% and 95.6%, indicating that they each comprised a single species. Group A and *L. mesenteroides* subsp. *dextranicum* JCM 9700^T, group B and *L. pseudomesenteroides* JCM 9696^T, and group C and *E. faecium* JCM 5804^T had a high DNA-DNA relatedness (>88.3%) and low DNA-DNA relatedness (<58.2%) against other groups and reference strains from the type strains of the previously described species, respectively. On the basis of DNA-DNA hybridization data, strains in groups A, B, C and D could be identified as belonging to *L. mesenteroides*

subsp. *dextranicum*, *L. pseudomesenteroides*, *E. faecium* and *L. plantarum*, respectively.

Discussion

LAB are the most important group of microorganisms in most cheese and are used as "natural" or "selected" starter cultures for several kinds of cheese. LAB play an important role during ripening, and contribute to the flavor and aroma of cheese (Stiles and Holzapfel, 1997). Many kinds of cheese are made from unpasteurized milk through traditional manufacturing procedures. Cheese has very varied and rich microflora. When cheese is produced from raw milk through traditional procedures, environmental microflora play a basic role in fermentation (Callon et al., 2006; Demarigny et al., 1997; Estepar et al., 1999; Marino et al., 2003). In the present study, the total LAB counts of Qula samples ranged between 10³ and 10⁷ g⁻¹ of FM, and were the dominant microflora in most Qula samples. Sengul and Cakmakci (Sengul and Cakmakci, 2003) found that the total number of LAB in helva cheese samples were 10⁵ to 10⁷, with an average of 7.6×10⁶ CFU g⁻¹. It is worthwhile pointing out that coliform bacteria exist in some Qula samples, so the significance of coliforms as a faecal indicator to as-

sess the hygienic quality of Qula appears to be questionable. This can be explained by the lack of available sanitary production methods. Although LAB play a crucial role, especially in the ripening of cheese due to lactic acid fermentation, it is impossible to prevent the growth of coliform, and especially proteolytic microorganisms. They either survive thermal treatments or enter the cheese and curd as post treatment contaminants (Prodromou et al., 2001).

Qula is produced from fresh yak milk using traditional methods, and is also made mainly by farmhouse cheese producers who manufacture only small amounts of cheese. Usually, Qula is conserved more than 2–3 years without any special preservation method at room temperature in local areas. Tibetans believe that Qula is safe and even has therapeutic virtues for diarrhea, even though it is usually collected, handled and transported in poor sanitary conditions and it is difficult to ensure that it attains a standard hygiene level. Such empirical observations were not scientifically substantiated. Few reports have been published on the microbial composition of Qula in spite of thousands of years of Tibetan experience in making Qula. Thus it is important to determine the microbial composition and LAB of Qula.

In the present study, all isolates were considered to be LAB as determined by culture on MRS agar, Gram stain appearance, catalase test, and lactate production from glucose. These LAB were characterized further by sugar fermentation assays using API 50CH strips. This led to the delineation of four groups of isolates, each one displaying a distinct similar carbohydrate fermentation pattern. The phenotypical characteristics of these isolates showed that groups A and B were typical *Leuconostoc*, and groups C and D were the enterococci and lactobacilli. As shown in Table 4, these isolates are easily distinguished from the previously described species for the type strains of *Leuconostoc*, *Enterococcus* and *Lactobacillus*. Therefore, these isolates could not be identified down to the species level on the basis of phenotypical characteristics.

In an effort to identify Qula LAB isolates at the species level, molecular phylogeny analysis was conducted and phylogenetic trees were constructed based on the 16S rRNA gene sequences from evolutionary distances by the neighbor-joining method. Following 16S rRNA gene sequence analysis, strains in groups B and C were placed in the genera *Leuconostoc* and *Enterococcus* in the phylogenetic tree and could be

identified as belonging to the *L. pseudomesenteroides* and *E. faecium*, respectively. Group A strains were identified as *L. mesenteroides* by forming a defined cluster with three subspecies of this type strain, as shown in Fig. 2. These strains were ascribed to the subspecies *mesenteroides*. Stackebrandt and Geobel (Stackebrandt and Geobel, 1994) reported that the 16S rRNA gene sequence analysis method, while very good at identifying the organisms by genus and species, cannot differentiate strains at the subspecies level and is therefore not the appropriate method to measure intraspecies relationships. Strains in group A were the nearest phylogenetic relatives to three subspecies of *L. mesenteroides*, with similarities exceeding 99%. The group D strains showed 16S rRNA gene sequence similarity of more than 99.5% to *L. plantarum* and *L. pentosus*. This is not surprising, given that the two species themselves have very similar 16S rRNA gene sequences that differ only by 2 bp (Hammes and Vogel, 1995). In fact, it is widely acknowledged that *L. plantarum* and *L. pentosus* belong to the same 16S rRNA phylogenetic group and can only be distinguished using phylogenetic analysis of sequences of the 16S-23S large space region (Ennahar et al., 2003), partial sequence of the *recA* gene (34) or DNA-DNA hybridization.

The DNA-DNA hybridization results demonstrated that strains in groups A, B, C and D could be assigned to *L. mesenteroides* subsp. *dextranicum*, *L. pseudomesenteroides*, *E. faecium* and *L. plantarum*, respectively. This is the first report of the identification of strains *L. mesenteroides* and *L. pseudomesenteroides* from Qula.

The natural fermentation processes in cheese were commonly dominated by species of *Lactobacillus* and *Lactococcus*, while some involved *Enterococcus* (Dervisoglu and Aydemir, 2007; Gurban et al., 2006; Khalid and Marth, 1990; López-Díaz et al., 2000; Torres-Llanez et al., 2006), and these species were the mostly dominant microbe in cheese. Few reports showed that *Leuconostoc* was the most dominant groups of LAB in cheese. In our study using biochemical and phylogenetic analyses, the majority of groups of LAB in Qula isolates were clearly shown to belong to the genera *Leuconostoc* (78%). The difference from other cheese was that *Lactobacillus* (15%) isolated from Qula was not a dominant genus. *E. faecium* (7%) was also a minor genus. Yet, a diversity of species was observed, since four other species were also

identified: *L. mesenteroides* subsp. *mesenteroides* (64%), *L. pseudomesenteroides* (7%), *E. faecium* (7%), *L. plantarum* (15%). Furthermore, heterofermentative species accounted for more than 70% of the total LAB microflora.

Although many studies have completed the characterization of the LAB in different cheese, our research provides the first approach to an up-to-date microbiological characterization of Qula. The isolation and identification of the LAB strains are considered the first step in the future research. The identification of fungi, yeast, acetobacter and bacilli isolated from Qula and the technological characterization of members of all these microbial groups are currently in progress.

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