The Journal of Microbiology (2011) Vol. 49, No. 5, pp. 721-728
DOI 10.1007/s12275-011-1062-9

Safety Evaluation *In Vitro* of *Enterococcus durans* from Tibetan Traditional Fermented Yak Milk

Jing Li[1], Fazheng Ren[2], Huiyong Gu[1], Xiaopeng Li[1], and Bozhong Gan[1]*

[1]*College of Food Science and Technology Engineering, Gansu Agricultural University, Lanzhou 730070, P. R. China*
[2]*Key Laboratory of Functional Dairy, Beijing Higher Institution Engineering Research Center of Animal Product, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, P. R. China*

(Received February 10, 2011 / Accepted May 18, 2011)

Despite its ubiquity in fermented dairy products, the safety of lactic acid enterococcal bacteria remains controversial. In this study, five *Enterococcus durans* strains – A1, A2, B1, B2, and C1 – were isolated from traditional fermented yak milk from Tibet. To evaluate the strains' safety, biogenic amine production, antibiotic resistance and presence of known virulence determinants were investigated. Strain A1 can produce biogenic amines for histamine, spermine, and spermidine (mean values: 8.64, 8.31, and 0.30 mg/L, respectively). Polymerase chain reaction amplification for Strain A1 found genes involved in expression of gelatinase (*gleE*), cytolysin (*cylA*, *cylB*, and *cylM*), sex pheromones (*ccf* and *cpd*) and cell wall adhesion (*efaA*). Strain A2 showed sensitivity or intermediate resistance to all tested antibiotics, and no virulence determinants except *gelE* and *ccf*, but did produce tyramine at a relatively high level (912.02 mg/L). Both strains B1 and B2 could produce histamine (10.43 and 10.56 mg/L, respectively), and showed vancomycin resistance; B1 also produced tyramine (504.02 mg/L). Strain C1 could produce all five biogenic amines tested in the study – putrescine, histamine, tyramine, spermine, and spermidine; concentrations were 6.51, 9.59, 205.85, 5.55, and 5.39 mg/L, respectively. All *E. durans* strains found in Tibetan traditional fermented yak milk thus offer potential risk.

***Keywords*:** fermented yak milk, *Enterococcus durans*, safety evaluation, biogenic amines, antibiotic resistance, virulence determinants

Consumption of fermented yak milk is part of Tibetan tribal culture. Yak milk is inoculated with a "natural" starter culture, prepared from milk from the previous milking. The raw milk is left to sour spontaneously at room temperature until it coagulates (Sun *et al.*, 2010). The microbial population inhabiting traditional fermented yak milk is very diverse; enterococci represent an essential part in this product, sometimes dominating over lactobacilli and lactococci therein (Beukes *et al.*, 2001; Ayad *et al.*, 2004). Although enterococci in food had previously been thought to result from faecal contamination, it is now accepted that enterococci naturally occur in raw milk and fermented milk products (Klein, 2003). *Enterococcus faecalis* and *Enterococcus faecium* are the species of enterococci most frequently isolated from dairy products; they have been thoroughly investigated by many researchers (Franz *et al*., 1999). *Enterococcus durans* is also frequently isolated, but its presence in dairy products has often been underestimated (Suzzi *et al.*, 2000; Andrighetto *et al.*, 2001; Cosentino *et al.*, 2004). Although traditional fermented yak milk, which contains *E. durans*, has long been a dietary mainstay of Tibetan herders, safety evaluation of *E. durans* has not been reported so far.

Despite enterococcal strains' well documented contributions to flavour, aroma and the ripening of fermented foods, they have not yet obtained GRAS (Generally Recognized as Safe) status (Centeno *et al.*, 1999). A possible negative aspect of enterococci is their ability to produce biogenic amines. Therefore, it is essential to evaluate the safety of enterococcal strains found in traditionally fermented foods (Connil *et al.*, 2002). Histamine and tyramine are the most studied biogenic amines, due to their vasoactive and psychoactive properties. In addition, some strains are typical opportunistic pathogens that cause human disease (Eaton and Gasson, 2001), which may be partly linked to the presence of virulence factors identified in enterococci, such as cytolysin (encoded by *cylA*) (Gilmore *et al*., 1994), gelatinase (encoded by *gelE*) (Su *et al.*, 1991), and enterococcal surface protein (encoded by *esp*) (Shankar *et al.*, 1999). The FAO-WHO has recommended that opportunistic virulence determinants be tested to document the safety of strains (FAO/WHO, 2002). Although enterococcal virulence factors are more common in clinical strains, they are also found in food-associated isolates (Eaton and Gasson, 2001; Franz *et al.*, 2001; Mannu *et al.*, 2003; Semedo *et al.*, 2003; Ben Omar *et al.*, 2004). Moreover, enterococcal virulence is strongly enhanced by their frequent resistance to commonly used antibiotics, which makes them effective opportunists in nosocomial infections (Giraffa, 2002).

Due to risks for transmission of potentially harmful enterococcal strains through the food chain, and the contribution of enterococci to spreading antibiotic resistance and to biogenic amine production, it is essential to evaluate the safety of strains found in foods, especially those used as starters or in other roles in fermented foods (Ogier and Serror, 2008).

* For correspondence. E-mail: ganbz@126.com; Tel.: +86-931-7631201; Fax: +86-931-7631201

In this context, this study aimed to evaluate the safety of *E. durans* strains that were isolated from traditional Tibetan fermented yak milk. Biogenic amine production, antibiotic resistance traits and incidence of virulence factors of each strain were investigated.

Materials and Methods

Strains isolation and identification

22 samples of traditional fermented yak milk, gathered from different households of herdsmen in Tibet, were collected aseptically in sterile bottles, kept in ice-box containers and carried to the laboratory for analyses within 4 h. From each sample, 1 ml was homogenized with 9 ml of 0.85% (w/v) sterile physiological saline to make an initial dilution (10^{-1}), and plated on a selective medium, kanamycin-aesculin-azide agar (oxoid) (Martin *et al.*, 2005). Purified strains were preserved in skim milk containing 20% glycerol and stored at -20°C until further study.

Enterococcal strains differ generally from other $Gram^{+}$ and $catalase^{-}$ cocci in several phenotypic traits, such as their ability to grow under moderately restrictive conditions (Hardie and Whiley, 1997). The following tests were carried out for presumptive identification of the isolates: observation of colony characteristics, Gram staining, catalase, tolerance to NaCl (2, 4, and 6.5%, w/v), and growth at 10°C and 45°C, and at pH 9.6 (Majhenic *et al.*, 2005; Sanchez Valenzuela *et al.*, 2009).

Potential enterococcal strains were identified by 16S rDNA sequencing. Total DNA of enterococcal strains was extracted using the TIANamp Bacteria DNA kit (Tiangen, China) according to the manual. Polymerase chain reaction (PCR) was performed using a final volume of 25 μl containing 1 U of 2× Taq polymerase (Tiangen), 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 3 mM $MgCl_2$, 500 μM of dNTPs, 1 μl of template, and 0.5 μM of each primer. Primers used were 5′-AGAGTTTGATC(A/C)TGGCTCAG-3′ and 5′-TACGG (C/T) TACCTTGTTACGACTT-3′ [synthetized by Invitrogen (Beijing) Co. Ltd] (Chun and Goodfellow, 1995; Loperena *et al.*, 2009). Amplification was performed in a PTC-200 thermocycler (Bio-Rad, USA) using the program followed by Loperenal *et al*. (2009). PCR products were purified using a Universal DNA Purification kit (Tiangen) and sequenced (AuGCT Bitechnology, China). The sequence was aligned in the National Center for Biotechnology Information (NCBI) database using BLASTn.

Biogenic amine production

To promote enzyme induction before the test, strains were subcultured 5 times in De Man Rogosa and Sharpe (MRS) broth containing 0.1% of each precursor amino acid (Aoboxing, China), comprising tyrosine, histidine, ornithine, and arginine in addition to supplementation with 0.005% of pyridoxal-5-phosphate (Aoboxing) (Bover-Cid and Holzapfel, 1999).

Regarding qualitative methods, most of the screening procedures use differential media containing pH indicators, such as bromocresol purple (Moller, 1954; Bover-Cid and Holzapfel, 1999). The decarboxylase medium allows a rapid preliminary selection of strains with low decarboxylase activity (Bover-Cid and Holzapfel, 1999). All strains were streaked in duplicate on decarboxylase medium plates according to Bover-Cid and Holzapfel (1999), with and without amino acids (as control). Isolates were assessed as decarboxylase medium-positive on the basis of colour transition from yellow to violet (Komprda *et al.*, 2010).

Decarboxylase medium presents some limitations in terms of sensitivity to biogenic amines (Bover-Cid and Holzapfel, 1999). Amine-forming capacity was quantitatively evaluated using high-performance liquid chromatography (HPLC). The strains, previously cultured in de MRS broth (containing precursor amino acid and pyridoxal-5-phosphate) were inoculated at 2% into a decarboxylase broth, for-

Table 1. PCR primers and product size for the detection of virulence and antibiotic determinants

Gene	Responsible for	Primer sequence (5′→3′)	Product size (bp)	Ref.
gelE	Hydrolysis of gelatin, collagen, hemoglobin	ACCCCGTATCATTGGTTT ACGCATTGCTTTTCCATC	419	Eaton and Gasson (2001)
esp	Immune evasion	TTGCTAATGCTAGTCCACGACC GCGTCAACACTTGCATTGCCGAA	933	Eaton and Gasson (2001)
cylA	Activation of cytolysin	TGGATGATAGTGATAGGAAGT TCTACAGTAAATCTTTCGTCA	517	Eaton and Gasson (2001)
cylB	Transport of cytolysin	ATTCCTACCTATGTTCTGTTA AATAAACTCTTCTTTTCCAAC	843	Eaton and Gasson (2001)
cylM	Posttranslational modification of cytolysin	CTGATGGAAAGAAGATAGTAT TGAGTTGGTCTGATTACATTT	742	Eaton and Gasson (2001)
cpd	Sex pheromones	TGGTGGGTTATTTTTCAATTC TACGGCTCTGGCTTACTA	782	Eaton and Gasson (2001)
cob	Sex pheromones	AACATTCAGCAAACAAAGC TTGTCATAAAGAGTGGTCAT	1045	Eaton and Gasson (2001)
ccf	Sex pheromones	GGGAATTGAGTAGTGAAGAAG AGCCGCTAAAATCGGTAAAAT	543	Eaton and Gasson (2001)
ace	Adhesion	AAAGTAGAATTAGATCCACAC TCTATCACATTCGGTTGCG	320	Dupre *et al*. (2003)
efaA	Antigen of bacteria endocarditis	GCCAATTGGGACAGACCTC CGCCTTCTGTTCCTTCTTTGGC	688	Ben Omar *et al*. (2004)
vanA	Vancomycin resistance	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	Dutka-Malen *et al*. (1995)
vanB	Vancomycin resistance	ATGGGAAGCCGATAGTC GATTTCGTTCCTCGACC	635	Dutka-Malen *et al*. (1995)

Table 2. 16S rDNA homology analysis of these isolates

Strains	The highest 16S rRNA homology strain	Homology (%)
A1	*E. durans* 16S ribosomal RNA gene, partial sequence, AF06100.1	100
A2	*E. durans* strain KLDS6.0402 16S ribosomal RNA gene, partial sequence, HM067028.1	100
B1	*E. durans* strain NM158-8 16S ribosomal RNA gene, partial sequence, HM218637.1	100
B2	*E. durans* strain NM78-2 16S ribosomal RNA gene, partial sequence, HM218342.1	100
C1	*E. durans* strain D-2 16S ribosomal RNA gene, partial sequence, GU299087.1	100

mulated as the screening medium, but without agar and containing 0.5% of each precursor amino acid (Bover-Cid and Holzapfel, 1999). After incubating at 37°C for 4 days, an equal volume of 10% trichloroacetic acid was added to 5 ml of fermenting broth and centrifuged at 4,000 rpm for 10 min after 1 h's extraction. Five standard biogenic amines (putrescine, histamine, tyramine, spermidine, spermine) (Sigma, USA) were prepared according to Shukla *et al.* (2010). Extracts were derived using Hwang's research (1997) with modifications of 1 g NaCl added to the mixture, and then incubated in a water bath at 60°C for 5 min to stop the derivative process (Yung-Hsiang *et al.*, 2005); 3 ml of ether were added and mixed by inversion, and the organic phases of the extracts were dried with nitrogen and redissolved in 1 ml carbinol. The filtered supernatant was kept at -20°C until HPLC analysis. Quantitative analyses of biogenic amines were carried out using a HPLC (SPD-20AT, Shimadzu, Japan) unit consisting of two pumps and a UV/VIS detector. Separation was achieved using a Kromasil C18 column (250 mm×4.6 mm, 5 μm). The gradient elution program started with an acetonitrile-water mixture (30:70, V/V), then proceeded to acetonitrile-water (60:40, V/V), with a flow-rate of 1 ml/min in 20 min. This was followed by the acetonitrile-water (90:10, V/V) at the same flow-rate for 8 min, then a decrease to acetonitrile-water (30:70, V/V) at 1 ml/min. The sample volume injected was 20 μl and the separation was monitored at 254 nm (Shukla *et al.*, 2010).

Assessment of antibiotic susceptibility

Antibiotic susceptibility of strains was determined on Mueller Hinton Agar (Majhenic *et al.*, 2005) using antibiotic discs (Tiantan Biological Products Co. Ltd, China). According to the antibiotic susceptibility standard for enterococci (CLSI, 2005), 17 antibiotics are listed in Table 4. After drying the surface, antibiotic discs were placed on the agar plate and incubated at 37°C. Zone diameters were recorded after 24 h incubation. *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 were used as quality control organisms. Antibiotic susceptibility levels for each enterococcus isolate was reported as resistant, intermediate-resistant or sensitive according to the CLSI (2005). Presence of vancomycin resistance genes *vanA* and *vanB* was also confirmed by PCR using primers described by Dutka-Malen *et al.* (1995) (Table 1).

PCR screening for virulence determinants

Total strain DNA was used in PCR reactions to detect the presence of genes: *gelE*, *cylA*, *cylB*, *cylM*, *esp*, *cpd*, *cob*, *ccf*, *ace*, and *efaA*. Primer sequences are listed in Table 1. Samples of DNA from *E. faecalis* ATCC 29212 and *E. faecalis* ATCC 19433 were used as positive controls. PCR amplification with primer pairs corresponding to particular virulence genes followed the procedure of Eaton and Gasson (2001) with modifications; the elongation time in 30 cycles was extended to 60 sec for *cob* and 30 sec for others, and the annealing temperature for *esp* and *gelE* was 60°C.

Results

Isolation and identification of strains

In the study, 17 strains were isolated from traditional fermented yak milk samples. Five Gram$^+$ catalase$^-$ strains that produced no gas, grew at 10°C and 45°C, grew in the presence of 6.5% NaCl and at pH 9.6, were considered as presumptive enterococci. All these five enterococcal strains were isolated from different samples. Nucleotide sequences of the 16S rDNA gene of all presumptive enterococcal isolates were analysed and identified in the NCBI database using BLASTn. Results of the BLAST nucleotide – nucleotide homology search indicated that five isolates belong to *E. durans* with 100% similarity (Table 2).

Biogenic amine production

The capacity to decarboxylate amino acids involved in generating biogenic amines was investigated. All isolates gave positive responses in the decarboxylation broth containing histidine, on both decarboxylase plates and in broth, which were recorded when a purple colour occurred around the colonies or in the decarboxylase broth, respectively. From all the strains assayed and confirmed by HPLC analysis, no false-positive reaction was observed.

The contents of biogenic amines of five *E. durans* strains are listed in Table 3. For strain A1, mean values of histidine, spermine and spermidine were found to be 8.64, 8.31, and 0.30 mg/L, respectively, while putrescine and tyramine was

Table 3. Quantified (mg/L broth) biogenic amine production by *E. durans* isolates

Sample No.	Concentration (mg/L)				
	Putrescine	Histamine	Tyramine	Spermine	Spermidine
A1	ND	8.64±0.12	ND	8.31±0.45	0.30±0.04
A2	6.66±0.01	10.07±0.06	912.02±104.38	3.45±0.00	ND
B1	12.86±0.01	10.43±0.08	504.02±0.12	29.72±0.13	ND
B2	5.98±0.00	10.56±0.27	ND	8.50±0.01	ND
C1	6.51±0.03	9.59±0.06	205.85±1.49	5.55±0.06	5.39±0.02

ND, not detected

Table 4. Antibiotic susceptibility for *E. durans* isolates

Antibiotic resistance		*E. durans* strains				
		A1	A2	B1	B2	C1
Quinolones	Levofloxacin	R	I	R	R	I
	Gatifloxacin	R	I	R	R	R
	Ciprofloxacin	R	I	R	R	R
	Norfloxacain	R	S	R	R	R
Glycopeptides	Vancomycin	S	S	I	R	S
	Teicoplanin	S	I	S	S	I
Macrolides	Erythromycin	I	I	R	I	I
Tetracyclines	Tetracycline	I	S	I	I	I
	Doxycycline	S	S	S	S	S
	Minocycline	S	S	I	I	I
Penicillins	Penicillin G	S	S	S	S	S
Others	Fosfomycin	S	S	S	S	S
	Rifampin	R	S	R	R	I
	Nitrofurantoin	S	S	S	S	S
	Chloramphenicol	S	I	R	S	R
	Streptomycin	S	S	S	S	S
	Gentamycin	S	S	S	S	S

R, resistant; I, intermediate resistant; S, sensitive

not detected. Both strains A2 and B1 produced putrescine, histamine, tyramine and spermine, at 6.66, 10.07, 912.02, and 3.45 mg/L for strain A2 and 12.86, 10.43, 504.02, and 29.72 mg/L for strain B1, respectively. Strain B2 produced putrescine, histamine and spermine at concentrations of 5.98, 10.56, and 8.50 mg/L, respectively, but did not produce tyramine and spermidine. Strain C1 produced all five biogenic amines tested – putrescine, histamine, tyramine, spermine, and spermidine – at concentrations of 6.51, 9.59, 205.85, 5.55, and 5.39 mg/L, respectively.

Antibiotic susceptibility

E. durans isolates were tested with 17 different antibiotics using the disc test method. According to the zone diameter interpretive standards for enterococci (CLSI, 2005), the prevalence of antibiotic resistance is shown in Table 4. All the isolates were sensitive to streptomycin, fosfomycin, doxycycline, nitrofurantoin, penicillin G, and chloramphenicol. Strain A2 possessed very low antibiotic resistance; it was sensitive or only intermediately resistant to all tested antibiotics. Strain A1 was sensitive to the tested glycopeptide antibiotics (vancomycin and teicoplanin) and penicillin G, but had intermediate resistance to erythromycin. Of the tetracyclines, strain A1 was sensitive to doxycycline and minocycline, and had intermediate

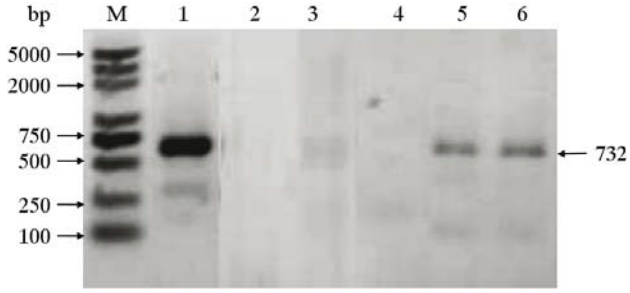

Fig. 1. PCR detection of the *vanA* gene conferring high-level resistance to vancomycin and related glycopeptides in *E. durans*. Lanes: M, Ladder; 1, positive control; 2, strain A1; 3, strain A2; 4, strain C1; 5, strain B1; 6, strain B2.

resistance to tetracycline. Strain A1 was sensitive to fosfomycin, nitrofurantoin, chloramphenicol, streptomycin, and gentamycin, but not rifampin, and was resistant to all four tested quinolones (levofloxacin, gatifloxacin, ciprofloxacin, and norfloxacain). Likewise, both strain B1 and B2 were considered resistant to these four quinolone antibiotics. Strain B1 was also resistant to erythromycin, rifampin, and chloramphenicol, but showed intermediate resistance to vancomycin, tetracycline, and minocycline. Strain B2 showed high resistance to the tested quinolones, and was also considered resistant to vancomycin and rifampin. Strain C1 was considered resistant to gatifloxacin, ciprofloxacin, norfloxacain, and chloramphenicol, but showed sensitivity or intermediate resistance to the other tested antibiotics.

E. durans strain B2 was identified as a vancomycin-resistant enterococcus; B1 had intermediate resistance. Incidence of vancomycin genes in tested strains was confirmed with PCR by the use of *vanA* and *vanB* specific primers. Two isolates (B1 and B2) gave the expected amplification products of 732 bp when amplified with specific primers for the *vanA* gene. PCR for the *vanA* gene results are shown in Fig. 1; PCR for the *vanB* gene yielded no PCR products (results not shown).

Virulence determinants

Incidence of virulence determinants among isolates is shown in Table 5. None of the isolates possessed all ten virulence determinants tested. *E. durans* strain A1 harboured seven virulence genes (*gelE*, *cylA*, *cylB*, *cylM*, *ccf*, *cpd*, and *efaA*). Results from PCR amplification of *cylA*, *cylB*, and *cylM* showed strain A1 to carry all these cytolysin genes. In con-

Table 5. Incidence of virulence determinants among *E. durans* strains

Strains	Virulence determinants									
	ace	*gelE*	*cylA*	*cylB*	*cylM*	*cob*	*ccf*	*cpd*	*esp*	*efaA*
A1	–	+	+	+	+	–	+	+	–	+
A2	–	+	–	–	–	–	+	–	–	–
B1	–	–	–	–	–	–	+	+	–	+
B2	–	+	–	–	–	–	+	–	–	+
C1	–	+	–	–	–	–	+	+	–	+

+, gene for virulence determinant detected
–, gene for virulence determinant not detected

trast, only *gelE* and *ccf* were detected in strain A2. Both strains B1 and B2 harboured three virulence genes; they all contained one of the sex-pheromone – encoding genes *ccf*, and the endocarditis enterococcal antigen gene *efaA*. In addition, B1 contained another sex-pheromone – encoding gene *cpd*, while B2 carried the gelatinase gene *gelE*. Genes *gelE*, *ccf*, *cpd*, and *efaA* were detected in C1.

Discussion

Enterococci are present in numerous fermented dairy products (Ogier and Serror, 2008), and *E. faecalis*, *E. faecium*, and *E. durans* are commonly found in milk products (Franz *et al*., 1999). In this study, five isolated strains were considered as presumptive enterococci by phenotypic identification. However, several phenotypic characteristics are not exclusive to enterococci and are shared by some strains of lactic acid bacteria such as lactococci (Murray, 1990; Franz *et al*., 1999). To avoid possible misclassification, genetic methods were used for reliable identification of enterococci at the species level; all five strains were identified to be *E. durans*.

The presence of enterococci in foods has been known for a long time, but has only recently been an object of food safety concern (Perez-Pulido *et al.*, 2006). These concerns are (Eaton and Gasson, 2001) controversial, partly due to the ability of enterococci to produce biogenic amines; this ability is reportedly a criterion for evaluating safety of enterococcal strains (Connil *et al*., 2002; Giraffa, 2002; Sanchez Valenzuela *et al*., 2009). Previous studies showed that tyramine was the only biogenic amine produced by enterococci in milk (Celano *et al.*, 1992; Giraffa *et al*., 1995). However, Sanchez Valenzuela *et al*. (2009) found that *E. faecium* in artisanal food showed a broader decarboxylating capacity, involving tyrosine, ornithine and histidine. In this study, five *E. durans* strains isolated from traditional fermented yak milk have different capacities to produce putrescine, histamine, tyramine, spermine, and spermidine. Low levels of biogenic amines in food are not considered a serious risk, but when consumed in excessive amounts, they may cause distinctive pharmacological, physiological and toxic effects (Tassoni *et al*., 2004). Different levels of histidines are regulated in different countries, e.g., 50 mg/L is proposed by the US Food and Drug Administration, while higher levels are recommended by the European Community, South Africa and Italy (100 mg/kg), and Germany (200 mg/kg) (Veciana-Nogues *et al.*, 1997; Auerswald *et al.*, 2006; Carelli *et al.*, 2007). All of the five *E. durans* strains can produce histamine (8.64 to 10.56 mg/L) but none of them were found to produce amounts of histamine that exceed the recommended action level by the Food and Drug Administration of 50 mg/L. In addition, three *E. durans* strains (A2, B1, and C1) showed the ability to produce tyramine, with amounts varying from 205-912 mg/L; no strains produced tyramine over the toxic level of 1,080 mg/L stipulated by Good Manufacturing Practice (Shalaby, 1996).

These results are in agreement with those obtained in previous studies showing high production of tyramine by enterococcal strains, and different enterococcal strains with various sources having different tyramine-producing capacities (Ruiz-Moyano *et al.*, 2009; Saaid *et al.*, 2009; Shukla *et al*., 2010). However, levels of tyramine formed in media were relatively high compared to those detected in fermented products such as kefir (Ozdestan and Uren, 2010). This may be due to the composition of decarboxylase medium, the incubation time, and the particular activity of the strains assayed (Bover-Cid and Holzapfel, 1999).

Biogenic amine production has been most extensively studied with respect to histamine and tyramine, probably the two most important biogenic amines of bacterial origin in food, due to their toxic effects (Bover-Cid and Holzapfel, 1999; Ozdestan and Uren, 2010). In this study, putrescine was also investigated since it may potentiate the toxicity of tyramine, and even serve as an indicator of poor hygiene in some food substrates (Matiné-Font *et al*., 1995). Sanchez Valenzuela *et al*. (2009) found that both *E. faecium* and *E. faecalis* showed decarboxylase activity on ornithine (the precursor amino acid of putrescine). In our study, all the strains gave a negative response in decarboxylase medium contained ornithine. However, some strains giving these false negatives were shown to be weak amine formers (concentrations were between 5.89-12.86 mg/L), and this might be due to the concentrations (5.89-12.86 mg/L) being too low to cause the pH shift and concomitant colour change (Bover-Cid and Holzapfel, 1999).

Antibiotic resistance trends among *E. faecium* and *E. faecalis* have been extensively reviewed (Franz *et al.*, 2003; Mannu *et al*., 2003; Peters *et al.*, 2003). Moreover, the occurrence of antibiotic resistance among dairy isolates seems to vary somewhat between studies, and is often strain- and region-dependent. There is no pattern that could classify enterococci with regard to their antibiotic susceptibility (Majhenic *et al*., 2005; Sanchez Valenzuela *et al*., 2009). Five *E. durans* strains were tested with 17 different antibiotics, and results revealed high incidences (except for A2) of resistance to levofloxacin, gatifloxacin, ciprofloxacin, norfloxacain, and rifampicin. Similarly, levofloxacin, ciprofloxacin, and rifampicin resistances have been reported at high frequency among *E. faecium* and *E. faecalis* isolates from artisanal foods (Sanchez Valenzuela *et al*., 2009). In this study, a high proportion (four of five) of *E. durans* isolates had intermediate resistance to tetracycline and erythromycin. Resistance or intermediate resistance to tetracycline and erythromycin were also seen in enterococcal strains by other researchers (Peters *et al*., 2003; Sanchez Valenzuela *et al*., 2009). Resistance to erythromycin is a matter of concern, because macrolides are common substitutes used in patients with a penicillin allergy (Barbosa *et al.*, 2009). Some researchers conclude that wide use of tetracycline in livestock may explain the prevalence of tetracycline resistance among enterococci (Busani *et al.*, 2004; Belicova *et al.*, 2007).

Vancomycin resistance is of special concern because emergence of vancomycin-resistant enterococci in hospitals has led to serious infections that cannot be treated with conventional antibiotic therapy (Bhardwaj *et al.*, 2008). In our study, *E. durans* B1 had intermediate resistance to vancomycin while B2 was resistant to it. The most frequent transferable vancomycin-resistant phenotypes are *vanA* and *vanB* (Cetinkaya *et al.*, 2000; Giraffa, 2002). Results showed that *E. durans* B1 and B2 have *vanA* genes, and these were the same strains resistant to vancomycin in antibiotic susceptibility tests. The fit between the phenotypic and the genetic tests indicates that the *vanA* genes present in the genome of these strains are phenotypically expressed as the acquired kind of vancomycin

resistance (Dupre *et al.*, 2003; Mannu *et al*., 2003).

An additional negative aspect of enterococci is that they are opportunistic pathogens (Franz *et al*., 1999; Bhardwaj *et al*., 2008). Eaton and Gasson (2001) identified distinct trends in the occurrence of virulence determinants in starter, food, and medical strains. Enterococci from cheese have been reported to carry multiple virulence traits (Majhenic *et al*., 2005). In this study, relevant factors for colonization of human tissues, such as the collagen adhesion gene (*ace*) and enterococcal surface protein gene (*esp*), were not detected in any of the isolates. Mannu *et al*. (2003) found that the collagen adhesion gene (*ace*) has always been described in *E. faecalis*. Likewise, the absence of surface protein gene (*esp*) is important, as it is thought to promote adhesion, colonization and evasion of the immune system (Foulquie Moreno *et al.*, 2006), while Mannu *et al*. (2003) considered that the surface protein gene (*esp*) was absent from dairy isolates. The gelatinase gene (*gelE*) is well known to have a silent state, making its genotypic study important because it might remain undetected under laboratory conditions but be expressed *in vivo* (Eaton and Gasson, 2001; Semedo *et al*., 2003). Although the *gelE* gene has been shown to be present more frequently in clinical isolates than in non-infectious strains (Eaton and Gasson, 2001), most *E. durans* strains (except for strain B1) isolated from traditional fermented milk were positive for the *gelE* gene. Tests for *cylA*, *cylB*, and *cylM* revealed that only strain A1 carried all three virulence determinants. Similarly, Majhenic *et al*. (2005) found that 50% of *E. faecalis* isolated from Tolminc cheese harboured *cylA*, *cylB*, and *cylM*. The distribution of haemolysin genes (*cylA*, *cylB*, and *cylM*) among enterococci is described differently by many researchers (Perez-Pulido *et al*., 2006; Ruiz-Moyano *et al*., 2009; Sanchez Valenzuela *et al*., 2009). The absence of these three genes explains the lack of haemolytic activity among most of the *E. durans* strains tested, since expression of such genes is required for the biosynthesis of cytolysin (Gilmore *et al.*, 1994). Genes encoding the endocarditis enterococcal antigen (*efaA*) showed a very high incidence being present in four of the five isolates. Mannu (2003) showed that the *efaA* was present in 19 out of 40 dairy strains. Presence of the *efaA* gene was also observed by Eaton and Gasson (2001) at similar frequencies in starter *E. faecium*, which has a long record of safe use in food. As we know, only the *efaA* gene from *E. faecalis* has been shown to influence pathogenicity in animal models, the role of the *efaA* gene from *E. faecium* has not been clearly demonstrated (Singh *et al*., 1998). But northern blot analysis indicated that the expression of the *efaA* gene of *Enterococcal* strains was induced by growth of the cell in medium supplemented with human serum. Researchers indicated that significant sequence variation in the *E. faecium* and *E. durans* strains may result in functional differences in the *efaA* gene that affect pathogenicity. Alternatively, in these strains, other virulence factors may be required in conjunction with *efaA* (Eaton and Gasson, 2001).

In conclusion, *E. durans* exists naturally in traditional fermented yak milk. We found that there were seven different virulence determinants present in *E. durans* A1. Strain A2 can produce tyramine, and both strains B1 and B2 showed a *vanA* genotype that confers high-level resistance to vancomycin. Therefore, *E. durans* strains should be regarded with caution, as they may produce biogenic amines when the sources of required precursor amino groups are abundant, and offer a reservoir for virulence genes and resistant to antibiotics. Our results reinforce the concerns expressed elsewhere about the safety of enterococcal strains found in foods, and suggest a potential risk regarding Tibetan fermented yak milk.

Acknowledgements

The authors gratefully acknowledge the financial support received by the Ministry of Education New Century Outstanding Talent of China Plan (Item No.NCET-10-0015), the Ministry of Science and Technology of China (2009BADB9B06), Research Project of Beijing Municipality (D101105046010001) and Beijing Municipal Commission of Education Co-constructed Program.

References

Andrighetto, C., E. Knijff, A. Lombardi, S. Torriani, M. Vancanneyt, K. Kersters, J. Swings, and F. Dellaglio. 2001. Phenotypic and genetic diversity of enterococci isolated from italian cheeses. *J. Dairy Res.* 68, 303-316.

Auerswald, L., C. Morren, and A.L. Lopata. 2006. Histamine levels in seventeen species of fresh and processed South African seafood. *Food Chemistry* 98, 231-239.

Ayad, E.H.E., S. Nashat, N. El-Sadek, H. Metwaly, and M. El-Soda. 2004. Selection of wild lactic acid bacteria isolated from traditional Egyptian dairy products according to production and technological criteria. *Food Microbiol.* 21, 715-725.

Barbosa, J., V. Ferreira, and P. Teixeira. 2009. Antibiotic susceptibility of enterococci isolated from traditional fermented meat products. *Food Microbiol.* 26, 527-532.

Belicova, A., L. Krizkova, J. Krajcovic, D. Jurkovic, M. Sojka, L. Ebringer, and R. Dusinsky. 2007. Antimicrobial susceptibility of Enterococcus species isolated from slovak bryndza cheese. *Folia Microbiol. (Praha)* 52, 115-119.

Ben Omar, N., A. Castro, R. Lucas, H. Abriouel, N.M.K. Yousif, C. Franz, W.H. Holzapfel, R. Perez-Pulido, M. Martinez-Canamero, and A. Galvez. 2004. Functional and safety aspects of enterococci isolated from different Spanish foods. *Syst. Appl. Microbiol.* 27, 118-130.

Beukes, E.M., B.H. Bester, and J.F. Mostert. 2001. The microbiology of South African traditional fermented milks. *Int. J. Food Microbiol.* 63, 189-197.

Bhardwaj, A., R.K. Malik, and P. Chauhan. 2008. Functional and safety aspects of enterococci in dairy foods. *Indian J. Microbiol.* 48, 317-325.

Bover-Cid, S. and W.H. Holzapfel. 1999. Improved screening procedure for biogenic amine production by lactic acid bacteria. *Int. J. Food Microbiol.* 53, 33-41.

Busani, L., M. Del Grosso, C. Paladini, C. Graziani, A. Pantosti, F. Biavasco, and A. Caprioli. 2004. Antimicrobial susceptibility of vancomycin-susceptible and resistant enterococci isolated in italy from raw meat products, farm animals, and human infections. *Int. J. Food Microbiol.* 97, 17-22.

Carelli, D., D. Centonze, C. Palermo, M. Quinto, and T. Rotunno. 2007. An interference free amperometric biosensor for the detection of biogenic amines in food products. *Biosens. Bioelectron.* 23, 640-647.

Celano, G.V., C. Cafarchia, F. Buja, and G. Tiecco. 1992. Biogenic-amines determination in cheese. *Industrie Alimentari* 31, 764-768.

Centeno, J.A., S. Menendez, M. Hermida, and J.L. Rodriguez-Otero. 1999. Effects of the addition of *Enterococcus faecalis* in cebreiro cheese manufacture. *Int. J. Food Microbiol.* 48, 97-111.

Cetinkaya, Y., P. Falk, and C.G. Mayhall. 2000. Vancomycin-resistant enterococci. *Clin. Microbiol. Rev.* 13, 686-707.

Chun, J.S. and M. Goodfellow. 1995. A phylogenetic analysis of the genus nocardia with 16S ribosomal-RNA gene-sequences. *Int. J. Syst. Bacteriol.* 45, 240-245.

Clinical and Laboratory Standards Institute. CLSI. 2005. Performance Standards for Antimicrobial Susceptibility Testing; 15th Informational Supplement. *CLSI*, M100-S15 25.

Connil, N., Y. Le Breton, X. Dousset, Y. Auffray, A. Rince, and H. Prevost. 2002. Identification of the *Enterococcus faecalis* tyrosine decarboxylase operon involved in tyramine production. *Appl. Environ. Microbiol.* 68, 3537-3544.

Cosentino, S., M.B. Pisano, A. Corda, M.E. Fadda, and C. Piras. 2004. Genotypic and technological characterization of enterococci isolated from artisanal fiore sardo cheese. *J. Dairy Res.* 71, 444-450.

Dupre, I., S. Zanetti, A.M. Schito, G. Fadda, and L.A. Sechi. 2003. Incidence of virulence determinants in clinical *Enterococcus faecium* and *Enterococcus faecalis* isolates collected in Sardinia (italy). *J. Med. Microbiol.* 52, 491-498.

Dutka-Malen, S., S. Evers, and P. Courvalin. 1995. Detection of glycopeptides resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J. Clin. Microbiol.* 33, 24-27.

Eaton, T.J. and M.J. Gasson. 2001. Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Appl. Environ. Microbiol.* 67, 1628-1635.

FAO/WHO. 2002. Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food, London, Ontario, Canada, April 30 and May 1, 2002.

Foulquie Moreno, M.R., P. Sarantinopoulos, E. Tsakalidou, and L. De Vuyst. 2006. The role and application of enterococci in food and health. *Int. J. Food Microbiol.* 106, 1-24.

Franz, C., W.H. Holzapfel, and M.E. Stiles. 1999. Enterococci at the crossroads of food safety? *Int. J. Food Microbiol.* 47, 1-24.

Franz, C., A.B. Muscholl-Silberhorn, N.M.K. Yousif, M. Vancanneyt, J. Swings, and W.H. Holzapfel. 2001. Incidence of virulence factors and antibiotic resistance among enterococci isolated from food. *Appl. Environ. Microbiol.* 67, 4385-4389.

Franz, C., M.E. Stiles, K.H. Schleifer, and W.H. Holzapfel. 2003. Enterococci in foods – a conundrum for food safety. *Int. J. Food Microbiol.* 88, 105-122.

Gilmore, M.S., R.A. Segarra, M.C. Booth, C.P. Bogie, L.R. Hall, and D.B. Clewell. 1994. Genetic-structure of the *Enterococcus faecalis* plasmid pad1-encoded cytolytic toxin system and its relationship to lantibiotic determinants. *J. Bacteriol.* 176, 7335-7344.

Giraffa, G. 2002. Enterococci from foods. *FEMS Microbiol. Rev.* 26, 163-171.

Giraffa, G., G. Pepe, F. Locci, and E. Neviani. 1995. Hemolytic activity, production of thermonuclease and biogenic amines by dairy enterococci. *Ital. J. Food Sci.* 7, 341-349.

Hardie, J.M. and R.A. Whiley. 1997. Classification and overview of the genera Streptococcus and Enterococcus. *J. Appl. Microbiol.* 83, S1-S11.

Hwang, D.F., S.H. Chang, C.Y. Shiua, and T.J. Chai. 1997. High-performance liquid chromatographic determination of biogenic amines in fish implicated in food poisoning. *J. Chromatography B* 693, 23-29.

Klein, G. 2003. Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *Int. J. Food Microbiol.* 88, 123-131.

Komprda, T., P. Sladkova, E. Petirova, V. Dohnal, and R. Burdychova. 2010. Tyrosine- and histidine-decarboxylase positive lactic acid bacteria and enterococci in dry fermented sausages. *Meat Sci.* 86, 870-877.

Loperena, L., M.D. Ferrari, A.L. Diaz, G. Ingold, L.V. Perez, F. Carvallo, D. Travers, R.J. Menes, and C. Lareo. 2009. Isolation and selection of native microorganisms for the aerobic treatment of simulated dairy wastewaters. *Bioresour. Technol.* 100, 1762-1766.

Majhenic, A.C., I. Rogelj, and B. Perko. 2005. Enterococci from tolminc cheese: Population structure, antibiotic susceptibility and incidence of virulence determinants. *Int. J. Food Microbiol.* 102, 239-244.

Mannu, L., A. Paba, E. Daga, R. Comunian, S. Anetti, I. Dupre, and L.A. Sechi. 2003. Comparison of the incidence of virulence determinants and antibiotic resistance between *Enterococcus faecium* strains of dairy, animal and clinical origin. *Int. J. Food Microbiol.* 88, 291-304.

Martin, B., M. Garriga, M. Hugas, and T. Aymerich. 2005. Genetic diversity and safety aspects of enterococci from slightly fermented sausages. *J. Appl. Microbiol.* 98, 1177-1190.

Matiné-Font, A., M.C. Vidal-Carou, M. Izquierdo-Pulido, M.T. Veciana-Nogués, and T. Hernández-Jover. 1995. Les amines biogenes dans les aliments: leur signification, Leur analyse. *Ann. Fals. Exp. Chim.* 88, 119-140.

Moller, V. 1954. Distribution of amino acid decarboxylases in enterobacteriaceae. *Acta Pathol. Microbiol. Scand.* 35, 259-277.

Murray, B.E. 1990. The life and times of the Enterococcus. *Clin. Microbiol. Rev.* 3, 46-65.

Ogier, J.C. and P. Serror. 2008. Safety assessment of dairy microorganisms: The enterococcus genus. *Int. J. Food Microbiol.* 126, 291-301.

Ozdestan, O. and A. Uren. 2010. Biogenic amine content of kefir: A fermented dairy product. *Eur. Food Res. Technol.* 231, 101-107.

Perez-Pulido, R., H. Abriouel, N. Ben Omar, R. Lucas, M. Martinez-Canamero, and A. Galvez. 2006. Safety and potential risks of enterococci isolated from traditional fermented capers. *Food Chem. Toxicol.* 44, 2070-2077.

Peters, J., K. Mac, H. Wichmann-Schauer, G. Klein, and U. Ellerbroek. 2003. Species distribution and antibiotic resistance patterns of enterococci isolated from food of animal origin in Germany. *Int. J. Food Microbiol.* 88, 311-314.

Ruiz-Moyano, S., A. Martin, M.J. Benito, E. Aranda, R. Casquete, and M.D. Cordoba. 2009. Safety and functional aspects of preselected enterococci for probiotic use in iberian dry-fermented sausages. *J. Food Sci.* 74, M398-M404.

Saaid, M., B. Saad, N.H. Hashim, A.S.M. Ali, and M.I. Saleh. 2009. Determination of biogenic amines in selected malaysian food. *Food Chem.* 113, 1356-1362.

Sanchez Valenzuela, A., N. Ben Omar, H. Abriouel, R. Lucas Lopez, K. Veljovic, M. Martinez Canamero, M.K. Ljubisa Topisirovic, and A. Galvez. 2009. Virulence factors, antibiotic resistance, and bacteriocins in enterococci from artisan foods of animal origin. *Food Control.* 20, 381-385.

Semedo, T., M.A. Santos, M.F.S. Lopes, J.J.F. Marques, M.T.B. Crespo, and R. Tenreiro. 2003. Virulence factors in food, clinical and reference enterococci: A common trait in the genus? *Syst. Appl. Microbiol.* 26, 13-22.

Shalaby, A.R. 1996. Significance of biogenic amines to food safety and human health. *Food Res. Int.* 29, 675-690.

Shankar, V., A.S. Baghdayan, M.M. Huycke, G. Lindahl, and M.S. Gilmore. 1999. Infection-derived *Enterococcus faecalis* strains are enriched in *esp*, a gene encoding a novel surface protein. *Infect. Immun.* 67, 193-200.

Shukla, S., H.K. Park, J.K. Kim, and M. Kim. 2010. Determination of biogenic amines in Korean traditional fermented soybean paste (doenjang). *Food Chem. Toxicol.* 48, 1191-1195.

Singh, K.V., T.M. Coque, G.M. Weinstock, and B.E. Murray. 1998. *In vivo* testing of an *Enterococcus faecalis efaA* mutant and use of *efaA* homologs for species identification. *FEMS Immun. Med. Microbiol.* 21, 323-331.

Su, Y.A., M.C. Sulavik, P. He, K.K. Makinen, P.L. Makinen, S. Fiedler, R. Wirth, and D.B. Clewell. 1991. Nucleotide-sequence of the gelatinase gene (*gelE*) from *Enterococcus faecalis* subsp.

liquefaciens. Infect. Immun. 59, 415-420.

Sun, Z., W. Liu, W. Gao, M. Yang, J. Zhang, L. Wu, J. Wang, B. Menghe, T. Sun, and H. Zhang. 2010. Identification and characterization of the dominant lactic acid bacteria from kurut: The naturally fermented yak milk in qinghai, China. *J. Gen. Appl. Microbiol.* 56, 1-10.

Suzzi, G., M. Caruso, F. Gardini, A. Lombardi, L. Vannini, M.E. Guerzoni, C. Andrighetto, and M.T. Lanorte. 2000. A survey of the enterococci isolated from an artisanal italian goat's cheese (semicotto caprino). *J. Appl. Microbiol.* 89, 267-274.

Tassoni, A., M.A. Germana, and N. Bagni. 2004. Free and conjugated polyamine content in Citrus sinensis Osbeck, cultivar barsillino N.L. 92, a Navel orange, at different maturation stages. *Food Chem*. 87, 537-541.

Veciana-Nogues, M.T., A. Marine-Font, and M.C. Vidal-Carou. 1997. Biogenic amines as hygienic quality indicators of tuna. Relationships with microbial counts, ATP-related compounds, volatile amines, and organoleptic changes. *J. Agric. Food Chem.* 45, 2036-2041.

Yung-Hsiang, T., K. Hsien-Feng, L. Tsong-Ming, C. Hwi-Chang, S.S. Chou, I.W. Cheng, and H. Deng-Fwu. 2005. Determination of histamine in canned mackerel implicated in a food borne poisoning. *Food Control.* 16, 579-585.