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Incidence of Virulence Determinants and Antibiotic Resistance in Lactic Acid Bacteria Isolated from Iranian Traditional Fermented Camel Milk (Chal)

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ABSTRACT: Lactic acid bacteria, including lactobacilli, enterococci, leuconostoc and weissella species isolated from Iranian traditional fermented camel milk (Chal) were assessed for the incidence of virulence determinants (*gelE*, $efaA_{fm}$, $efaA_{fs}$, *ace*, esp_{fs}, *cylM*, *cylA and cylB)*, sensitivity to various antibiotics and virulence phenotypes. The incidence of virulence genes was determined by polymerase chain reaction and antibiotic susceptibility was tested by disk diffusion method. The results of this study indicated that all of the strains harbor at least two or more of the virulence genes. The most frequent virulence genes detected among tested strains were *cylB*, *gelE* and $efaA_{fs}$. All strains showed no β-hemolysis while tyrosine decarboxylase activity and gelatinase production were observed in enterococcus and leuconostoc strains. Majority of strains were resistant to Polymyxin B and kanamycin. Lactobacillus strains including *L. paraplantarum*, *L. kefiri*, *L. paracasei*, *L. plantarum* and *Weissella cibaria* were resistant to both vancomycin and kanamycin. The possibility of transferring the antibiotic resistance and virulence genes to other starter and commensal strains makes the usage of these strains in food and dairy products controversial without required safety assessments.

Keywords: *Enterococcus faecium, Fermented Milk, Lactic Acid Bacteria, Safety Assessment, Virulence Determinants.*

Introduction

Fermented dairy products have been an important part of the diet of human along history (Adimpong *et al.,* 2012). Camel milk, which is used as fresh, raw milk or fermented milk, is an essential part of the human diet in the arid regions of the world (Moslehishad *et al.*, 2013).Fermented camel milk (Chal) is a very popular beverage in TurkmanSahra, Golestan province, Iran. It is a spontaneous mixed fermented dairy product which includes yeasts and lactic acid bacteria (LAB) as the predominant microflora. Chal includes a wide range of different bacterial strains that may have safety issues which should be considered at the time of consuming. Because of their frequent occurrence in fermented food products, long history of safe use and being part of the human commensal microflora, LAB are believed to be GRAS (Generally Recognized as Safe) by many scientific groups (Zhou *et al*., 2000; Choi *et al.*, 2005). Also, according to EFSA (European Food Safety Agency) most LAB species are included in the QPS (Qualified Presumption of Safety) list (Munoz-Atienza *et al.*, 2013). However, there have been some reports on

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infectious diseases such as endocarditis, bacteremia and urinary tract infections mostly in patients with underlying illnesses that some lactobacillus strains have been involved. Moreover, the acquisition of vancomycin resistance by enterococcus strains specially *Enterococcus faecium* resulted in the emergence of vancomycin resistant enterococci (VRE) which could cause infection in hospitalized patients (Patel, 2003). Today, safety is a priority in food and dairy industry and an important step for introducing the traditional products for industrial production.Therefore, safety assessment is an important criterion to ensure the safety and quality of these fermented products (Tan *et al.*, 2013). In the recent decades spread of antibiotic resistant microorganisms led to worldwide concern, therefore the antibiotic resistance patterns should be tested to document the safety of strains. Because of their wide usage as starter culture and probiotics, lactobacilli could transfer the antibiotic resistance genes to other LAB or pathogens (Bernardeau *et al.*, 2008). There is a poor document about virulence factors and antibiotic resistance of Lactobacillus strains in the literature. LAB group also includes enterococci species that occur or deliberately added to fermented foods and they may have an important role in the development of sensory characteristics (Bhardwaj *et al*., 2010; Inoğlu and Tuncer 2013; Togay *et al*., 2010). Due to previous reports, *E. faecium* and *E. faecalis* predominantly cause disease, notably nosocomial infections in human. Virulence factors such as hyaluronidase, aggregation substance, cytolysine and enterococcal surface protein encoded by *hyl, agg, cyla* and *esp* genes, respectively have been detected in enterococcal strains (Inoğlu and Tuncer, 2013). The differences between pathogen and safe strains of enterococci and other LAB are not clear. These strains are introduced as live cultures, especially by some traditional fermented food products (Eaton and Gasson, 2001). Therefore an individual evaluation of safety of potential probiotic strains should be considered. The aim of this study is to investigate the safety of strains of LAB isolated from common traditional fermented camel milk (Chal) in terms of the presence of virulence genes and antibiotic susceptibility.

Materials and Methods

- *Study of Potential Virulence Determinants*

Overnight-culture of LAB strains isolated from Chal samples (Soleymanzadeh *et al*., 2016) was streak-plated on de Man, Rogosa, and Sharpe (MRS) broth (Scharlau, Spain) and incubated at 37°C anaerobically in a candle jar for 24 h. Bacterial culture (10-20 mg) was collected by centrifugation at 5000 ×g for 10 min. Genomic DNA was extracted using the cinnapure DNA extraction kit (SinaClone Co., Tehran, Iran) and following the manufacturer's instructions. DNA was stored at −20°C and used for all PCR reactions mentioned in this study.

Total DNA of the 11LAB strains was used to detect the presence of virulence genes, including *gelE, efaA*$_{fm}$, *efaA*$_{fs}$, *ace, esp*$_{fs}$, *cylM, cylA andcylB*. The primers are listed in Table 1. Oligonucleotide primers were obtained from Sinaclone Co. (Tehran, Iran). The positive control strain for detection of virulence genes was *E. faecalis* PTCC 1778. PCR-amplifications were performed using bacterial DNA in 20µL reaction mixtures with 100 ng of extracted DNA, 1.25µM of each primer, 0.2 mM of each dNTP, buffer 1× 1.5 mM $MgCl_2$ and 0.75 U of Taq DNA polymerase (Sina Clone Co., Tehran, Iran). Samples were subjected to an initial cycle of denaturation (95°C for 5 min), followed by 35 cycles of denaturation (94°C for 1 min), annealing (48 to 60°C for 1 min) and elongation (72°C for 1 min), ending with a final extension step at 72°C for 10 min (Eaton and Gasson, 2001) in an Eppendorf Master cycler thermal cycler (Eppendorf, Hamburg, Germany). PCR products were

detected by electrophoresis for 30 min at 90 V on 1.5% (w/v) agarose gels stained with DNA loading dye (Thermo Fisher Scientific, USA), and visualized under UV light with the Gel Doc 1000 documentation system (Bio-Rad, Madrid, Spain). The molecular size markers used were 1kb DNA Ladder (Thermo Fisher Scientific, USA).

- *Determination of antibiotic susceptibility*

Antibiotic susceptibility of the 11 strains was determined by commercially antibiotic-containing disks (Padtan-teb, Iran) on MRS agar plates previously seeded with approximately 1×10^5CFU/ml of each isolates. The antibiotics tested were ampicillin(AM) (10 µg), kanamycin (K) (30 µg), ciprofloxacin (CIP) (5 µg), gentamicin (GM) (10 µg), penicillin G (P) (10 mcg), polymixin B (PB) (300 u), rifampicin (RIF) (5 µg), tetracycline (TE) (30 µg), and vancomycin (V) (30 µg). The zone of inhibition was measured after overnight incubation of the plates at 37°C. The tests were carried out in triplicate order.

- *Gelatinase and Hemolysine production*

For determination of gelatinase production the strains were grown in MRS broth at 37°C for 24 h. These cultures were streaked onto Todd-Hewitt agar plates containing 30 g of gelatin (Biolife, Milano, Italy), incubated overnight at 37°C, and placed at 4°C for 5 h before examination for zones of turbidity around the colonies, indicating hydrolysis. In order to investigate hemolysin production, the strains were streaked onto layered fresh sheep blood agar plates and grown for 1-2 days at 37°C. Clear zones around the colonies indicated the presence of β-hemolysis (Eaton and Gasson, 2001).

- *Decarboxylase activity*

The decarboxylase test for the production of biogenic amines was carried out by incubation of LAB strains for 2-5 days on 37°C on improved decarboxylase medium (Bover-Cid and Holzapfel, 1999) contained 1 g/l final concentration of the following amino acids as precursors: histidine, tyrosine, lysine and ornithine (Himedia, Mumbai, India). After incubation time the purple color around the colonies indicated the production of biogenic amino acids.

Table 1. Primers for detection of the virulence genes (Eaton and Gasson 2001)

Gene	The Role of Product	Sequence (5´-3´)	Product Size (bp)
gel E	gelatinase	F: ACCCCGTATCATTGGTTT R: ACGCATTGCTTTTCCATC	419
efaA$_{fa}$	cell wall adhesion	F: AACAGATCCGCATGAATA R: CATTTCATCATCTGATAGTA	735
efaA$_{fs}$	cell wall adhesion	F: GACAGACCCTCACGAATA R: AGTTCATCATGCTGTAGTA	705
ace	collagen adhesion	F: AAAGTAGAATTAGATCCACAC R: TCTATCACATTCGGTTGCG	350
esp$_{fs}$	cell wall-associated protein	F: TTGCTAATGCTAGTCCACGACC R: GCGTCAACACTTGCATTGCCGAA	933
cylM	cytolysine	F: CTGATGGAAAGAAGATAGTAT R: TGAGTTGGTCTGATTACATTT	742
cylA	cytolysine	F: TGGATGATAGTGATAGGAAGT R: TCTACAGTAAATCTTTCGTCA	517
cylB	cytolysine	F: ATTCCTACCTATGTTCTGTTA R: AATAAACTCTTCTTTTCCAAC	843

Results and Discussion

- *Virulence determinants*

The presence of virulence genes in LAB strains are listed in Table 2. A total of 11 strains of lactic acid bacteria isolated from Chal samples including *L. paraplantarum, L. kefiri, L. paracasei, L. gasseri, L. plantarum, E. faecium* (4 strains), *W. cibaria and Leu. lactis.* All strains were screened for the presence of eight virulence genes. All of the strains were found to harbor at least two or more of the tested virulence determinants. Among the eight tested virulence genes, the strains of *E. faecium* were positive for the four following virulence factors: *gelE, $efaA_{fm}$, $efaA_{fs}$* and *cylB*. Togay *et al*., (2010) investigated *E. faecium* and *E. faecalis* strains isolated from fermented Turkish foods and their results showed that majority of tested *E. faecium* strains carried *gelE, $efaA_{fm}$, $efaA_{fs}$* and some other virulence determinants. Eaton and Gasson (2001) studied *E. faecium* strains and found that all *E. faecium* starter and food strains were clear of virulence determinants except for *$efaA_{fm}$* and only one of the medical *E. faecium* strains was positive for *gelE*. In another study, *gelE* and *$efaA_{fm}$* were found to be some of the most frequently presented in *E. faecium* and *E. faecalis* strains (Inoğlu and Tuncer, 2013).The role of *$efaA_{fm}$* has not yet been identified. Although there is sequence variation in the *E. faecium* strains that could cause functional differences in the *efaA* adhesion and influence pathogenicity (Bhardwaj *et al*., 2010). Although β-hemolytic activity was not found in any of the strains, all of the tested strains were positive for *cylB*. It might be due to the absence of *cylA* and *cylM* or presence of silent genes in our strains. Mannu *et al*., (2003) reported that none of their tested *E. faecium* strains harbored the gene for *gelE*. In the present study, eight out of 11 strains were positive for *gelE* gene (Figure 1). Gelatinase production was detected in enterococci and leuconostoc strains. Although some of lactobacillus strains and *W. cibaria* SM09 carried *gelE* gene, none of them showed gelatinase activity phenotypically in biochemical test. Therefore silent *gelE* gene might have occurred in these strains. Regarding the obtained results and the literature, it is difficult to say that *E. faecium* strains are safe for use in food and dairy industry. The incidence of virulence factors is strain specific and in spite of clinical strains, food and starter strains of *E. faecium* have a lower potential for pathogenicity. However, the possibility of transferring the antibiotic resistance and virulence genes to other starter and commensal strains still exists (Franz *et al*., 2001). Considering the obtained results, there is a possibility of entering the enterococcal contamination to traditional fermented products like Chal and it might be due to lack of hygiene.

Table 2. Presence of virulence genes among strains isolated from (fermented camel milk) Chal

Strains	Genotype	Relevant Phenotype	
L. paraplantarum SM01	*ace^+, $cylB^+$*	$^1Gel^-$, $^2Hly^-$	$^3Tyr^-$, $^4Lys^-$, $^5Orn^-$, $^6His^-$
L. kefiri SM02	*$efaA_{fs}{}^+$, $cylB^+$*	Gel^-, Hly^-	Tyr^-, Lys^-, Orn^-, His^-
E. faecium SM03	*gel E^+, $efaA_{fm}{}^+$, $efaA_{fs}{}^+$, $cylB^+$*	Gel^+, Hly^-	Tyr^+, Lys^-, Orn^-, His^-
L. paracasei SM04	*$efaA_{fs}{}^+$, ace^+, $cylB^+$*	Gel^-, Hly^-	Tyr^-, Lys^-, Orn^-, His^-
L. gasseri SM05	*gel E^+, $efaA_{fs}{}^+$, $cylB^+$*	Gel^-, Hly^-	Tyr^-, Lys^-, Orn^-, His^-
L. plantarum SM06	*gel E^+, $efaA_{fs}{}^+$, ace^+, $cylB^+$*	Gel^-, Hly^-	Tyr^-, Lys^-, Orn^-, His^-
E. faecium SM07	*gel E^+, $efaA_{fm}{}^+$, $efaA_{fs}{}^+$, $cylB^+$*	Gel^+, Hly^-	Tyr^+, Lys^-, Orn^-, His^-
E. faecium SM08	*gel E^+, $efaA_{fm}{}^+$, $efaA_{fs}{}^+$, $cylB^+$*	Gel^+, Hly^-	Tyr^+, Lys^-, Orn^-, His^-
W. cibaria SM09	*gel E^+, $efaA_{fs}{}^+$, ace^+,$esp_{fs}{}^+$, $cylB^+$*	Gel^-, Hly^-	Tyr^-, Lys^-, Orn^-, His^-
Leu. lactis SM10	*gel E^+, $efaA_{fm}{}^+$, ace^+, $cylB^+$*	Gel^+, Hly^-	Tyr^+, Lys^-, Orn^-, His^-
E. faecium SM11	*gel E^+, $efaA_{fm}{}^+$, $efaA_{fs}{}^+$, $cylB^+$*	Gel^+,Hly^-	Tyr^+, Lys^-, Orn^-, His^-

1Gel and 2Hly are respectively gelatinase and cytolysine activities. 3Tyr, 4Lys, 5Orn and 6His are respectively for Tyrosine, Lysine, Ornithine, and Histidine decarboxylase activities.

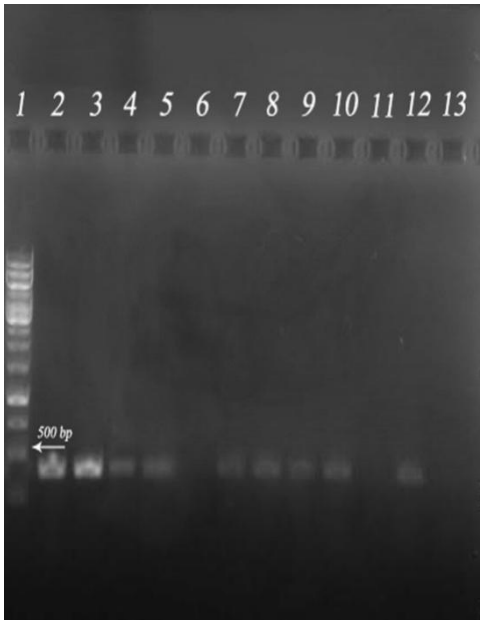

Fig. 1. Multiplex PCR for *gelE* gene. lane 1, molecular weight marker; lane 2,PTCC 1778 reference strain; lane 3, *E. faecium SM03* ; lane 4, *E. faecium SM07*; lane 5, *E. faecium SM08;* lane 6, *L. paraplantarum SM01;* lane 7, *E. faecium SM11,*; lane 8,*L. gasseri SM05*; lane9, *L. plantarum SM06;* lane 10, *W. cibaria SM09*; lane 11, *L. kefiri SM02*;lane 12, *Leu. lactis SM10*; lane 13, *L. paracasei SM04.*

Lactobacillus strains have been found to harbor some of the virulence genes tested in this study. All of the strains were positive for *cylB* and except *L. paraplantarum* the other strains harbored $efaA_{fs}$. Moreover, *gelE* has been detected in *L. gasseri* and *L. plantarum*. While all enterococcus strains were negative for *ace* gene, it has been shown that *L. paraplantarum, L. paracasei* and *L. plantarum* carried this gene. However the results indicated that all lactobacillus strains were negative for $efaA_{fm}$, *cylA*, *cylM* and esp_{fs}. According to our knowledge, there is a lack of reports on the safety assessment of lactobacillus strains in the literature while according to obtained results it seems to be essential to assess the strains present in traditional fermented foods.

- *Antibiotic susceptibility*

Antibiotic susceptibility of the strains to various antibiotics was determined by a disc diffusion method and results are summarized in Table 3. Most of the strains were found to be resistant to ploymyxin B and kanamycin. No vancomycin resistance was detected among enterococci, indicating that these strains did not acquire resistance determinants for vancomycin.VRE are the possible food reservoirs in the dispersion of vancomycin resistance genes in the environment (Giraffa, 2002) and it seems that the abundance of vancomycin resistance between enterococci is increasing in Europe and cause difficulties in the treatment of infections with them (Arias *et al*., 2010). Our results were consistent with Bhardwaj *et al*. (2010) who reports that in contrast with clinical strains, 90% of enterococci isolated from dairy products were susceptible to vancomycin. In another study Teuber *et al*. (1999) reported low incidence of vancomycin resistance between enterococci isolated from European cheese. Adimpong *et al*. (2012) showed that all of the tested LAB strains were resistant to kanamycin and vancomycin that was consistent with our results. Although incidence of tetracycline resistance is quite widespread between lactic acid bacteria, all of the strains were sensitive

to tetracycline. As shown in table 3, among the strains, *W. cibaria* SM09, *Leu. Lacis* SM10 and *L. paracasei* SM04 were resistant to gentamicin. Also *L. gasseri* SM05 and *L. plantarum* SM06 were resistant to ciprofloxacin. In this study, it was indicated that four of the lactobacillus strains and *W. cibaria* SM09 were resistant to both vancomycin and kanamycin and this multiple antibiotic resistance to aminoglycosides and vancomycin makes some concerns, especially among lactobacillus strains and their usage as starter culture. However, resistance against vancomycin could be an intrinsic property according to Ammor *et al*. (2007) that reported vancomycin resistance of *Lactobacillus, Pediococcus* and *Leuconostoc* species is as a result of the absence of D-Ala-D-lactate in their peptidoglycan which is the target of vancomycin and it is different from vancomycin resistant in enterococci encoded by transmissible plasmids. Kanamycin resistance in *E. faecium* is due to the frequent presence of aminoglycoside 6´-acetyltransferase which is an intrinsic property. Therefore gentamycin and streptomycin are recommended aminoglycosides for a synergistic therapy in combination with a cell wall agent for enterococci (Arias *et al*., 2010).

Amino acids histidine, lysine and ornithine were not decarboxylated by any of the strains. However, all of the enterococcus strains and *Leu.lactis* SM10 decarboxylated tyrosine.

Conclusion

The results of this study suggested that according to the potential risk factors, traditional fermented foods need safety assessments in order to investigate their bacterial strains for harboring the virulence genes and transmissible resistance against antibiotics. The majority of strains isolated from Chal including lactobacillus, enterococci, leuconostoc and weissella carried virulence genes and exhibited resistance against some of the antibiotics such as vancomycin and kanamycin. Additionally, some of these strains showed gelatinase and tyrosine decarboxylase activity. Regarding the findings of our study, there is a possibility of entering the clinical strains of enterococci to fermented foods because of the lack of hygiene. It should be born in mind that ingestion of large numbers of these strains through consumption of Chal might be a potential risk factor for consumer health and according to the obtained results consideration of these strains as starter culture requires more safety evaluation.

- *Evaluation of dacarboxylase activity*

Table 3. Antibiotic susceptibility determination

Strains	Antibiotic Sensitivity								
	[1]P	[2]PB	[3]V	[4]TE	[5]AM	[6]K	[7]GM	[8]RIF	[9]CP
L. paraplantarum SM01	[10]S	[11]R	R	S	S	R	[12]I	S	S
L. kefiri SM02	S	S	R	S	S	R	S	S	R
E. faecium SM03	S	R	S	S	S	R	S	S	S
L. paracasei SM04	S	R	R	S	S	R	R	S	S
L. gasseri SM05	S	R	S	S	S	S	S	S	R
L. plantarum SM06	S	R	R	S	S	R	S	S	R
E. faecium SM07	S	R	S	S	S	R	S	S	S
E. faecium SM08	S	R	S	S	S	R	S	S	S
W. cibaria SM09	S	R	R	S	S	R	R	S	S
Leu. lactis SM10	S	R	S	S	S	R	R	S	S
E. faecium SM11	S	R	S	S	S	R	S	S	S

Abbreviations: 1P: Penicillin, 2PB: Polymyxin B,3V: Vancomycin,4: Tetracyclin,5AM: Ampicillin, 6K:Kanamycin, 7GM: Gentamicin,8 RIF: Rifampicin, 9CP: Ciprofloxacin, 10S: Sensitive, 11R: Resistant, 12I: Intermediate.

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