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Research Article
Virulence Profiles in *Enterococcus* spp. Isolated from Raw Buffalo's Milk in South Brazil

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Abstract

Background and Objective: The buffalo milk consumption and derivatives have increased significantly in the last year due to the healthy food demand. Enterococci play a beneficial role during the maturation of some cheese and sausages; they have been used as probiotics in humans and animals. On the other hand, they are indicators of fecal contamination and are frequently associated with foodborne illnesses by biogenic amines. The aim of this study was to evaluate the presence of virulence profiles in enterococci strains isolated from raw buffalo's milk samples. **Materials and Methods:** Seventy-nine enterococci species were selected which previously identified by conventional biochemical methods. The strains were submitted to genotypic identification using genus-specific and species-specific primers. Strains were tested for the presence of virulence genes (*agg*, *ace*, *gelE*) by PCR, their ability to form biofilms and to produce the enzyme gelatinase by phenotypic methods. The optical density (OD) of bacterial biofilms was quantified in a spectrophotometer. **Results:** The phenotypic and genotypic identification were similar in more than 96% of the strains. The frequency of *ace* (96 vs. 10.34%) and *gelE* (96 vs. 17.24%) genes were higher in *E. faecalis* than in *E. faecium*, while the *agg* gene was detected only in *E. faecalis* strains (26%). The *in vitro* biofilm ability was observed in both strains; however, it was superior among *E. faecalis* (90%) than in *E. faecium* (24.1%). The presence of *gelE* and the activity of gelatinase were not fully concordant. **Conclusion:** It was concluded that the presences of enterococci harboring virulent factors in raw buffalo's milk suggest a situation of risk for the community, since enterococci are opportunist pathogens. The ability to form biofilm is important for food safety and the protection of public health. In this sense, the present study sought to collaborate with the status quo of scientific knowledge to improve safety and quality of the food for human consumption.

Key words: Enterococci, raw buffalo's milk, virulence genes, genotypic identification, biofilm formation

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The buffalo milk has a high concentration of proteins and fats, low cholesterol content, than other animals[1]. Buffalo milk is the raw material used to prepare buffalo mozzarella cheese and other dairy products. The mozzarella cheese is the main product, being the largest part of the production of milk intended for their manufacture[1,2]. In Brazil, the mozzarella cheese is produced from the pasteurized buffalo's milk, however some production system follows Italian process and work with raw milk[3,4]. This process has the purpose to ensure the particular organoleptic characteristics and inherent to the product, despite the importance of pasteurization[3].

Enterococcus spp. are lactic acid bacteria, producing bacteriocins and are widely distributed in nature, present in soils, waters, foods, plants and vegetables[5-7]. Moreover, this genera is commonly found in the gastrointestinal tract of humans and animals[8-11]. In addition, they are tolerant to high concentrations of salts, pH variations and a wide temperature range. This rusticity has been demonstrated that some enterococcal strains can survive at temperatures of milk pasteurization[7].

The genus *Enterococcus* contains over 50 recognized species and *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*) are the most frequent species isolated from clinical and food[7]. Among the species, *E. faecalis* is recognized to have a well elucidated arsenal of virulence factors, when compared to the others, possessing multiple virulence determinants, which reinforces the evidence of pathogenicity[12,13]. The presence of enterococci in foods can be an indicator of fecal contamination in the production and/or processing of food and also serve as reservoirs of genes for resistance, which can spread through the food chain, thus contributing to the spread of antibiotic resistance in the human population. Epidemiological studies show that by their nature opportunist, enterococci have emerged as pathogens associated with nosocomial infections, which may be related, in part, to antibiotic resistance and the presence of virulence determinants[7,14,15].

Virulence determinants are factors which enable the bacteria to colonize, invade, prevent the defense system and cause tissue damage to the host[16]. Among the virulence factors widely found in this genus, highlights the gelatinase enzyme (encoded by the *gelE*); aggregation substance of *Enterococcus* (encoded by *agg* gene), adhesion of collagen from *E. faecalis* (encoded by *ace*) and the ability to form biofilm[17].

Studies that assess the microbiological quality of buffalo's milk are still scarce[6], due to the difficulty in obtaining samples. In this scenario, the knowledge of strains circulating in food, contributes to a better understanding of the opportunistic behavior of these microorganisms in the food chain. Given this, the aim of this study was to evaluate the presence of virulence genes, the ability to form *in vitro* biofilm and to produce the gelatinase in enterococci isolates from raw buffalo's milk samples in South Brazil.

MATERIALS AND METHODS

***Enterococcus* strains:** The study occurred in March, 2014 with 79 enterococci (51 *E. faecalis*; 23 *E. faecium*; two *E. duran* and three *Enterococcus* spp.), isolated from raw buffalo's milk samples in South Brazil[6], during June-August, 2012, were selected (Table 1). All the strains have already been subjected to conventional biochemical tests by Prichula *et al.*[6]. The strains were maintained in a solution of 10% (w/v) of skim milk (Difco) and 10% (v/v) glycerol (Neon Comercial Ltda.), frozen at -20°C, in the Coccus Gram-positive Laboratory and Molecular Microbiology at Federal University of Health Sciences of Porto Alegre (UFCSPA).

It is important to note that the 79 bacteria used in this study, 13.9% (11/79) showed resistance to nitrofurantoin, 12.7% (10/79) to tetracycline, 1.3% (1/79) to chloramphenicol, 1.3% (1/79) to streptomycin, 1.3% (1/79) to norfloxacin and 1.3% (1/79) to erythromycin. Forty-seven isolates (59.5%) showed intermediate resistance to norfloxacin, 58.2% (46/79) to ciprofloxacin, 51.9% (41/79) to erythromycin and 6.3% (5/79) to the nitrofurantoin[6].

Molecular identification of genus by PCR: All strains were confirmed as genus by polymerase chain reaction (PCR) using a genus-specific primer of $tuf_{Enterococcus}$ gene, according to the protocols described by Ke *et al.*[18] (Table 2).

Table 1: Genotypic and phenotypic profile of enterococci isolated from buffalo's milk evaluated in this study

	Percentage of strains identified	
Species	Phenotypic[1] N (%)	Genotypic[2] N (%)
E. faecalis	64.56* (51/79)	63.30 (50/79)
E. faecium	29.11 (23/79)	36.70 (29/79)
E. durans	2.53** (2/79)	0
Enterococcus spp.	3.80*** (3/79)	0

[1]Phenotypic identification by Prichula *et al.*[4]. [2]Identification based on species-specify PCR. *One strain was reclassified as *E. faecium* after PCR. **Species reclassified as *E. faecium* after PCR. ***Species identified as *Enterococcus* spp. and identified an *E. faecium* after PCR

Table 2: Primers and annealing temperatures used in the PCRs carried out in this study

Genes	Primers	Sequence (5'-3')	PCR product (bp)	Tm (°C)	Reference
tuf$_{Enterococcus}$	Ent 1	TACTGACAAACCATTCATGATG	112	54	Ke *et al.*[18]
	Ent 2	AACTTCGTCACCAACGCGAAC			
ddl E. faecium	F	TTGAGGCAGACCAGATTGACG	658	54	Cheng *et al.*[19]
	R	TATGACAGCGACTCCGATTCC			
ddl E. faecalis	F	ATCAAGTACAGTTAGTCTTAATTA	475	54	Depardieu *et al.*[20]
	R	ACGATTCAAAGCTAACTGAATCAGT			
gelE	gelE1	AGTTCATGTCTATTTTCTTCAC	402	56	Mannu *et al.*[21]
	gelE2	CTTCATTATTTACACGTTTG			
ace	ACE1	AAAGTAGAATTAGATCCACAC	320	56	Mannu *et al.*[21]
	ACE2	TCTATCACATTCGGTTGCG			
agg	TE3	AAGAAAAAGAAGTAGACCAAC	1553	56	Eaton and Gasson[14]
	TE4	AAACGGCAAGACAAGTAAATA			

Genomic DNA was extracted by chemical analysis method following the method described by Donato[22]. The PCR was carried[18] out in a reaction volume of 25 µL, containing 100 ng of genomic DNA, 1.5 mM $MgCl_2$, 10 µM of each primer *tuf* gene, 200 µM of dNTPs, 1U *Taq* DNA polymerase and reaction buffer 1X. Amplification was carried out in an Eppendorf Mastercycler Personal 5332 Thermocycler (Eppendorf®) according to the following program: 95°C for 3 min followed by 35 cycles of 95°C for 30 sec, 54°C for 30 sec, 72°C for 1 min, followed by final extension at 72°C for 7 min. The PCR products were analyzed by electrophoresis gel with 1.5% agarose, stained with ethidium bromide solution.

Species confirmation by polymerase chain reaction: A specie-specific PCR assay followed was used to confirm the phenotypic identification. Listed in Table 2 are the primers. The conditions of the PCR were previously described[18]. The DNA fragment amplified by PCR was analyzed in 1.5 % (w/v) agarose gels.

Virulence profile

Detection of virulence genes by PCR: The presence of the *agg* (aggregation substance of *Enterococcus*), *ace* (adhesion of collagen from *E. faecalis*) and *gelE* (expression of gelatinase enzyme) genes followed as described by Medeiros *et al.*[23]. The PCR for *ace*, *agg* and *gelE* genes was performed in 25 µL total volume and conditions have been described previously[14,18,21]. The DNA fragments amplified by PCR were analyzed in 1.5% (w/v) agarose gels.

Gelatinase activity: The gelatinase activity was performed in tubes, following the protocol described by Marra *et al.*[24].

***In vitro* biofilm formation on polystyrene microplate:** All strains were tested for the ability to form biofilm *in vitro* by using the crystal violet (CV) protocol [25]. Briefly, the experiment was divided into three stages: First, the bacteria were previously inoculated on agar plates containing Brain Heart Infusion (BHI-Himedia) and incubated to 35-37°C for 18 h. After the growth, the colonies were resuspended in saline and adjusted scale of 0.5 McFarland standards (approximately 10^8 CFU mL^{-1}). The wells of sterile 96-well flat-bottomed polystyrene microplates were filled with 180 µL of Tryptic Soy Broth (TSB-Himedia) supplemented with 1% glucose and 20 µL of bacterial suspension (containing approximately 10^8 CFU mL^{-1}). Afterwards, the plates were incubated for 18 h at 36°C. The optical density (OD) of bacterial biofilms was quantified in a using 492 nm wavelength in a spectrophotometer (Anthos 2010 Microplate Reader). The OD of each strain was determined by the arithmetic mean of the absorbance of the wells and this value was compared with the mean absorbance of negative controls (ODnc). The strains were separated into categories using the O.D values, as: non-biofilm (ODs<ODnc), weak biofilm (ODnc<ODs<2.ODnc), moderate biofilm (2.ODnc<ODs<4.ODnc) and strong biofilm producers (4.ODnc<ODs). All biofilms assays were run in 8 replicates and repeated three times. *Staphylococcus epidermidis* American type culture collection 35984 classified as a strong adherent was used as the positive control and TSB plus 1% glucose as a negative control[25].

RESULTS

Genotypic identification of *Enterococcus* spp. isolates from raw buffalo's milk: The results between phenotypic and genotypic identification were similar in more than 96% of the strains. Only three strains (two *E. durans* and one *E. faecalis*) had discordant between phenotypic and genotypic identification (Table 1). However, for the genotypic identification those strains were reclassified as atypical *E. faecium*. These isolates had phenotypic traits similar to *E. durans* and *E. faecalis,* indicating the need to further evaluate their taxonomic position. The three strains are phenotypic characterized as *Enterococcus* spp. were genotypically identified as *E. faecium*.

Table 3: Distribution of virulence genes among *E. faecalis* and *E. faecium* isolated from buffalo's milk in South Brazil

	No. (%) of strains positive to		
Species (n)	*ace*	*agg*	*gelE*
E. faecalis (50)	48 (96.00)	13 (26.00)	43 (86.00)
E. faecium (29)	3 (10.34)	0%	5 (17.24)
Total (79)	51 (64.55)	13 (16.45)	48 (60.75)

Analysis of virulence profiles

Frequency of the virulence genes by PCR and detection of gelatinase activity: All isolates were evaluated for the presence of virulence genes by PCR and the results are presented in Table 3. The virulence genes were more frequent in *E. faecalis* when compared to *E. faecium*. In the present study, the *ace* gene was detected in 96% (48/50) of *E. faecalis* and 10.34% (3/29) of *E. faecium*. The *agg* gene was detected only in *E. faecalis* strains (26%).

The *gelE* gene was detected in 86% (43/50) and 17.24% (5/29) and *E. faecium*, respectively. The gene *gelE* encodes a gelatinase and of the 79 strains evaluated, 28 showed gelatinase activity, in addition this phenotype was observed only in *E. faecalis.* For some strains, the gelatinase activity *in vitro* was not correlate with the presence of *gelE* genes. Analysis of congruence between gelatinase and *gelE* pointed out that 30% (15/50) *E. faecalis* and 17.24% (5/29) *E. faecium* positive to *gelE* gene were unable to degraded gelatin as a substrate.

***In vitro* biofilm formation ability of enterococci isolates from raw buffalo's milk:** Biofilm formation of *E. faecalis* and *E. faecium* isolates from raw buffalo's milk samples were assayed and categorized based on the OD_{492}. The biofilm ability was observed in high frequency among *E. faecalis* (90%) than in *E. faecium* (24.1%). Of the 50 *E. faecalis* strains, 10 (20%), 32 (64%) 3 (6%) and 5 (10%) were classified as strong, moderate, weak and non-biofilm formers, respectively. Among the 29 *E. faecium*, only 4 (13.8%) were strong biofilm former and two (10.3%) moderates and 22 (75.9%) non-biofilm former.

Since the expression of gelatinase may be influencing the formation of biofilm, it was observed that more than half of *E. faecalis* strains gelatinase positive (56%) were the strains classified as moderate (64%) and strong (20%) biofilm former.

DISCUSSION

Identification of *Enterococcus* spp. by PCR: Of the 79 enterococci tested, three strains had discordant between phenotypic and genotypic identification and were reclassified as atypical *E. faecium*. Atypical enterococci have already been identified in strains isolated from food origin[5]. In addition, Jahan *et al.*[26] reported that negative results of biochemical tests can be explained the metabolic diversity of organisms in this genus. Nevertheless, genotypic assays are quicker, practical and more sensitive when compared with the biochemical tests. Knudtson and Hartman[27] and Moore *et al.*[28], showed that identification based on molecular tools are more accurate detection, avoid long periods of incubation of culture and eliminate phenotypic results ambiguous.

Analysis of virulence factors

Virulence genes and detection of gelatinase activity: The acquisition of virulence factors enables the bacteria to survive and reproduce in competitive environments. Differences in the frequency of virulence genes were observed among *E. faecalis* and *E. faecium* isolates from raw buffalo's milk samples. *Enterococcus faecalis* are known to harbored more virulence genes than *E. faecium*. Cariolato *et al.*[13], Gomes *et al.*[29] and Barbosa *et al.*[30], have demonstrated a high frequency of multiple virulence factors in *E. faecalis* strains, however the same pattern was not observed in *E. faecium* strains. Mannu *et al.*[21] pointed out that *E. faecium* is a species generally free of known virulence factors, although there are some references related to occasional virulence determinants in this isolated species[14,31,32].

Virulence genes detected in this study were *ace*, *agg* and *gel*. A high frequency of *ace* gene in *E. faecalis* (96%) was reported in this study. Gomes *et al.*[29], in agreement with this study, detected this gene in food and clinical isolates, belonging to species other than *E. faecalis*. However, Cariolato *et al.*[13] described the gene *ace* only in *E. faecalis* strains isolated dairy products. The *ace* gene encodes a collagen adhesion protein involved in mediating adherence to epithelial. The *ace* gene has been associated with infections caused by *E. faecalis*, antibodies against *ace* have been almost always found in serum from patients with endocarditis[15].

The *agg* gene which encodes an aggregation substance that facilitates cell adhesion was detected only in *E. faecalis* isolates. This result is in agreement with several researchers that described *agg* gene only in *E. faecalis* strains[14,28]. Eaton and Gasson[14] when analyzing *Enterococcus* foodborne isolates, isolated clinical and used as starter cultures, established the *agg* gene only in strains of *E. faecalis*.

In the present study, *gelE* gene was detected in *E. faecalis* and *E. faecium*. Moraes *et al.*[33] reported a high frequency of *gelE* in *Enterococcus* strains isolated from raw milk and cheese in Minas Gerais state, Brazil. The gelatinase is an

enzyme capable of hydrolyzing substrates such as gelatin, collagen, casein, hemoglobin and other small biologically active peptides[14,34]. The expression of gelatinase in *Enterococcus* isolates from raw buffalo's milk samples can be explained by the fact that these samples are rich in casein and the production of gelatinase may be a selection mechanism for growth allowing the microorganisms using the casein as a source of amino acids. The presence of *gelE* genes did not always correlate with gelatinase. According to Marra *et al.*[24], the presence of the gene *gelE* is not necessarily correlated with the phenotype of gelatinase. This result may be justified by four factors: (a) The loss of gelatinase activity during laboratory manipulation[11], (b) Presence of silent genes, (c) Low levels of down regulation of gene expression or (d) Due to the influence of environmental factors in the expression of the enzyme[14].

Biofilm formation in enterococci isolates from raw buffalo's milk: In the food industries, biofilm has become a problem, mainly related to the fact that microorganisms in food processing equipment are resistant to sanitizers and sources of contamination. Ability to form biofilm was observed in *E. faecalis* and *E. faecium* isolated from buffalo's milk samples; however, this ability was more frequently in *E. faecalis* strains. The results report in the literature about the formation of biofilm by enterococci in foods, corroborate with the results demonstrate here[23,29]. Microorganisms growing on food surfaces and in processing environments can cross-contaminate and cause post-processing contamination. The milk industries pay special attention to the cross-contamination during food process. Many outbreaks have been associated with biofilm. The clinical relevance of biofilms formed by enterococci is, mainly, their significantly higher antibiotic resistance relative to their free-living[17].

CONCLUSION

In conclusion, the data indicate that enterococci isolated from buffalo's milk samples may act as reservoirs of virulence genes, which can spread these genes to human's microbiota through the food chain. The ability to form biofilm by the strains is important for food safety and the protection of public health, since enterococci within the biofilms are protected from sanitizer increasing the likelihood of survival and subsequent contamination of food. Additional investigation of these microorganisms in food, as well as, the characterization and identification of virulence profile is necessary to ensure the quality of the food. The increasing importance of *Enterococcus* as a nosocomial opportunistic and at the same time their utilization in the food industry as starter cultures and flavor aggregators, researches are necessary to ensure safety and quality of the food for human consumption.

SIGNIFICANCE STATEMENTS

This study evaluated virulence profiles and enterococci strains isolated from raw buffalo's milk samples. Since the buffalo milk consumption and derivatives have increased significantly in the last year, this study suggests an alert situation for the community, as enterococci are opportunist pathogens. The ability to form biofilm is important for food safety and the protection of public health. Additional investigation of these microorganisms in food, as well as, the characterization and identification of virulence profile is necessary to ensure the food security and the quality of the food.

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