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Safety Evaluation of Enterococcal Strains Isolated from Dairy Products and Clinical Samples Using RT-PCR

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Abstract: Enterococci are part of the dominant microbiota of several dairy products, which can be used in the dairy industry as starter or probiotic cultures. However, they are also implicated in severe multi-resistant nosocomial human infections. In this study, phenotypic tests using API 20 S strip were used for species identification of 60 and 55 enterococcal strains isolated from dairy products and clinical samples, respectively. Tests for production of gelatinase, hyaluronidase and haemolysin were done with all enterococcal isolates, whereas molecular determination of virulence markers (genes of *gelE*, *hyl*, *cylA*, *ASA 1*and *ESP* revise in the text) using RT-PCR technique and biofilm formation were checked only for *E. faecium* and *E. faecalis* isolates. Our obtained results depicted that *E. faecium* (56.6 %) was the predominant species isolated from dairy products, followed by *E. faecalis* (36.6%), *E. gallinarumm* (3.3%) and (1.6%) of both *E. casseliflavous* and *E. hira.* In contrast, *E. faecalis* (76.3%) was the predominant enterococcal strain identified from human clinical isolates followed by *E. faecium* (21.8%) and (1.8%) *E. gallaniurm.* Different and distinct patterns of incidence of virulence determinants were found for *E. faecalis* and *E. faecium* strains. In general, the incidence of virulence traits was lower among *E. faecium* strains than among *E. faecalis* from dairy products. Also, our results showed that the incidence of virulence factors was highest among clinical enterococcal isolates, followed in decreasing order by dairy strains, suggesting that the dairy strains have a lower potential for pathogenicity. Finally, these results reinforce and suggest that the use of *Enterococcus* spp. in dairy industry as starter or probiotics culture requires careful safety evaluation.

Key words: Enterococci · Dairy products · Probiotic cultures · Infect6ion · PCR

INTRODUCTION

Enterococci are lactic acid bacteria (LAB) that are important in environmental, food and clinical microbiology. These bacteria may play an important beneficial role in the various traditional food products in the world and may also be successfully used as probiotics. It is well known that enterococci are a relevant component of the bacterial population of a variety of cheeses produced from raw or pasteurized milk [1]. Enterococci may contaminate the milk either directly from animal feces or indirectly from a contaminated water source, milking equipment or the bulk storage tank [2].

Several studies have indicated that strains of enterococci may have a positive influence on the production and ripening of cheeses, namely they disseminate in the environment, their heat, salt, acid and temperature tolerance and their biochemical properties (proteolytic and lipolytic activity, production of flavor components) significantly contribute to the formation of specific organoleptic and quality characteristics of ripened chesses [3].

Moreover, certain strains of *E. faecium*, *E. faecalis* and *E. durance* have been proposed as a part of defined starter cultures for different cheese and other fermented dairy products, while some *E.faecium* and *E.faecalis* strains demonstrated anti-listeria activity and can be therefore used as protective cultures in cheese technology [2]. On the other hand, their presence is unwanted in certain cheeses and in processed meat products in which they may cause spoilage problems. However, although fermented foods containing enterococci have a long history of safe use, the presence of enterococci in food is of considerable concern for the

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food industry and consumers. Indeed, enterococci are considered as emerging pathogens of humans and are often associated with hospital acquired infections [4].

In spite of the beneficial activities of enterococci, they may present an emerging threat as human pathogens associated with nosocomial infections. Particularly, strains of *E. faecalies* as a predominant species and to, a lesser extent *E. faecium* have been reported to be involved in human pathogenesis [2, 4].

One of the reasons for the rise of nosocomial infections related to enterococci might be their ability to develop resistance against a wide variety of antibiotics. Enterococci have also been recognized as potential nosocomial pathogens causing infections such as bacteremia and endocarditis. Several studies have recently shown that they may harbor putative virulence factors such as cytolysins, gelatinase, serine protease, hyaluronidase, aggregation substance, extracellular surface protein and other adhesions involved in binding to host cell, biofilm formation and plasmid encoded pheromones [5-7]. Antibiotic resistance, in combinations with the above mentioned virulence factors; determine that enterococci can not be considered as generally recognized safe (GARS) microorganisms [8].

In the Directive 2000/54/EC [9] of the European parliament concerning risks of exposure to biological agents, the genus enterococcus is allocated as a whole into risk-group 2, which includes microorganisms harboring potential virulence factors. The differentiation of apparently safe and non-safe enterococci strains is not simple, especially because virulence genes can be easily exchanged between strains [10, 11].

Real-time PCR is the most recent, rapid and widely applied technology for direct quantification of cells in mixed samples. Real time PCR is increasingly being used for direct detection and quantification of pathogens in foods and environmental or clinical samples [11].

Therefore, in order to select an enterococci strain as a potential starter culture candidate for dairy products especially in cheese making, not only the technological characteristics but also the reliable identification of enterococci and their susceptibility to clinically relevant antibiotics together with the incidence of virulence determinants should be thoroughly investigated as well.

To this regard, our study was devoted to investigate the safety and virulence factors profiles of both *E. faecalies* and *E. faecium* strains isolated from dairy products (especially cheeses) and clinical human samples using rapid and advanced real time PCR techniques.

MATERIALS AND METHODS

Ssamples and Strains: A total of 115 enterococci isolates was used in this study. Sixty strains were isolated from dairy products (cheeses and milk) collected from different markets by the technique described previously by Lopes *et al.* [12]. Fifty-five clinical isolates was obtained from human infections in several hospitals without any known epidemiological relationship. All dairy and clinical isolates were identified phenotypically by the API 20 strep system (bioMerieux).

Detection of Haemolysin, Gelatinase and Hyaluronidase: Haemolysin and gelatinase production were detected as described by Eaton and Gassonand [5] and Franz *et al.* [13], respectively.

Biofilm Assay: The ability of the enterococci isolates to form a biofilm on an abiotic surface was quantified as described by Toledo-Arana *et al.* [14]

RT-PCR for Detection of Virulence Factors:

DNA Preparation and Extraction: DNA for the detection of genes coding for enterococci virulence factors was extracted by taking 200 ul of each isolates grown overnight in MRS broth, pelleted by centrifugation at 13000 g for 5 min. The pellets were washed twice with sterile water in a clean 1.5 micro centrifuge tube and re-pelleted by centrifugation. Total DNA was extracted from washed cell pellets by fully automated MagNA pure LC instrument and MagNA pure LC DNA isolation kits (Roche Diagnostics, Germany) according to manufacturer's instructions.

Primers for Screening of Virulence Genes by Real-Time PCR: Five oligonucleotides primers pairs used to amplify the specific genes of the following enterococcal virulence determinaents, *gelE* (Gelatinase), *hyl* (Hyaluronidase), *cylA* (Cytolysin), *asal* (Aggregation Substance) and *esp* (Enterococcal surface protein) were purchased from TibMolibiol (Germany). The expected amplicon size and primer sequences were as previously published by Vankerckhoven *et al.* [15] and are listed in Table(1).

Real-Time PCR (By Sybr Green I Formate): Virulence factors profile for the identified enterococcal isolates were determined on previously prepared (eluted and stored) template DNA of such isolates by using Real-Time Light Cycler PCR and Light Cycler FastStart Master SYBR Green 1 kit (Roche Diagnostics GmbH) which contain all components of the PCR master mix except primers and

Table 1: PCR primers and products for detection of virulence determinants of the examined enterococci

Gene	Virulence factor	Primer name	Oligonucleotide sequence (5' to 3')	Product size (bp)
gelE	Gelatinase	GEL11	TATGACAATGCTTTTTGGGAT	213
		GEL12	AGATGCACCCGAAATAATATA	
hyl	Hyaluronidase	HYLn1	ACAGAAGAGCTGCAGGAAATG	276
		HYLn2	GACTGACGTCCAAGTTTCCAA	
cylA	Cytolysin	CYT1	ACTCGGGGATTGATAQGC	688
		CYT11b	GCTGCTAAAGCTGCGCTT	
asa 1	Aggregation substance	ASA11	GCACGCTATTACGAACTATGA	375
		ASA12	TAAGAAAGAACATCACCACGA	
esp	Enterococcal surface protein	ESP14F	AGATTTCATCTTTGATTCTTGG	510
		ESP12R	AATTGATTCTTTAQCATCTGG	

template DNA. Five PCR master mixes consisting of different primer sets were prepared. The base master mix consisted of 8.6µl of H_2O (PCR grade), 2.4µl of 4mMgcl_2 and 2µl of LC FastStart DNA Master SYBR Green 1. Two µl of 0.5-1mM primer mix for each virulence factor were added to the base mix. PCR was performed in a final volume of 20µl (in LC capillary) consisting of 15µl master mix and 5µl of purified template DNA. Following the experimental protocol mentioned in the kit's manual instruction, pre-incubation at 95°C for 10 minutes, mixtures were amplified by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 55°C for 5 seconds and elongation at 72°C for 10 seconds. Melting curve analysis of the amplified product was performed by one cycle at 95°Cfor 10 seconds, 65°C for 15 seconds and rising the temperature to 95°C by rate of 0.1°C per second (temperature transition). The fluorescence profiles generated from each sample were cleared in channel 1 (F1). Comparing the fluorescence intensity of the sample with positive and negative controls in each run as well as the results were confirmed by the melting curve analysis for each sample.

RESULTS AND DISCUSSION

The role of enterococci in food is still unclear and increasing interest on the epidemiology of these bacteria has been derived from the ability of some antibiotic - resistant strains to colonize and cause disease in hospitalized patient worldwide [17]. In this sense, the identification of the enterococci related to specific clinical events and even their distribution in foods is of crucial importance to understand the epidemiology of this genus.

In our study, out of 60 dairy enterococci, the following isolates were identified phenotypically at species level; 22 (36.6%) *E. faecalies*, 34 (56.6%) *E. faecium*, 2(3.3%) E. gallinarumm, 1 (1.6%) *E. casseliflavous* and 1 (1.6%) *E. hira.* In contrast, *E. faecalis* (76.3%) was the predominant enterococcal strain identified from a total of 55 human clinical isolates followed by E. faecium (21.8%) and E.gallaniurm (1.8%) (Table 2).

The above mentioned data depicted that most common species found in examined samples of dairy products and clinical source were *E. feacium* and *E. faecalis.* The prevalence of these two species in dairy products has also been reported in other surveys [18-20]. A higher prevalence of E. faecium in dairy products (especially cheese) may be attributed to their resistance to heat, extreeme salinity and harsh conditions during ripening of cheese [2, 21].

Results of conventional phenotypic tests and hemolytic characterization performed with predominate isolates obtained are presented in Table 3. In dairy products isolates, gelatinase was only produced by 9(40.9%) of *E. faecalis* isolates, while *E. faecalis* and *E. feacium* isolated from clinical samples produced gelatinase activity with percentage of 57.1 and 16.6 %, respectively.

ß-hemolytic activity was most prevalent among *E. faecalis* (22.7 and 14.3 %) when compared to *E. faecium* (0 and 8.3%) in both isolates from dairy products and clinical samples respectively. B. hemolytic isolates are considered undesirable in foods and their use as starters in food fermentation is not recommended [22].

Hyaluronidase activity was only detected in *E. feacalis* and *E. feacium* isolated from clinical source with percentage of 59.5 and 41.6 %, respectively.

Fifty-six dairy and 54 clinical strains were screened for the presence of five known virulence determinants by a rapid novel real-time PCR using SyBer Green I formate.

A summary of the incidence and distribution of these determinants in *E. faecalis* and *E. faecium* according to their origin is shown in Table (4).

The *gel* gene occurred in *E.faecalis* strains of clinical (83.3%) as well as of dairy (59 %) origin, while among the species of *E.faecium* the only gel positive strain occurred

Table 2: Phenotyping of *Enterococcus* isolates revealed from dairy products and clinical samples using API 20 strep??.

		Identified isolates									
		E. faecalis		*E. faecium*		*E. casseliflavous*		*E. gallinarum*		*E. hira*	
Source of sample	No. of isolates	No.	%	No.	%	No.	%	No.	%	No.	%
Dairy products	60	22	36.76	34	56.76	1	1.6	2	3.3	1	1.6
Clinical samples	55	42	76.43	12	21.8	0	0	1	1.8	0	0

Table 3: Biochemical and haemolytic characterization of the identified isolates using conventional methods other than API 20 strep.

							Haemolysis					
			Gelatinase		Hyaluronidase		α		ß		Γ	
Source of Sample	Type of Isolates	No. of Isolates	No. of +ve	%	No. of +ve	%	No.	%	No.	%	No.	%
Dairy products	*E. faecalis*	22	9	40.9	0	0	15	68.2	5	22.7	2	9.1
	E. faecium	34	0	0	0	0	30	88.2	0	0	4	11.8
Clinical samples	*E. faecalis*	42	24	57.1	25	59.5	5	11.9	6	14.3	31	73.8
	E. faecium	12	2	16.6	5	41.6	7	58.3	1	8.3	4	33.3

Table 4: Prevalence and genotyping distribution of some virulence determinants in some of the identified *Enterococcus* isolates by Real-Time PCR (using SYBR Green I format).

			+ve virulence determinants									
			Gel		*Hyl*		*Cyt*		*ASA*		*Esp*	
Source of sample	Type of isolates	No. of tested isolates	No.	%	No.	%	No.	%	No.	%	No.	%
Dairy products	*E. faecalis*	22	13	59	0	0	3	13.6	10	45.5	7	31.8
	E. faecium	34	3	16.7??	0	0	1	2.9	0	0	0	0
Clinical samples	*E. faecalis*	42	35	83.3	29	69.1	17	40.5	28	66.6	40	95.2
	E. faecium	12	2	16.6	5	41.6	3	25	5	41.6	9	75

Table 5: Frequency distribution and grading of identified *Enterococcus* isolates according to biofilm production. *

	E. faecalis				*E. faecium*			
	Dairy(n=22)		Clinical(n=42)		Dairy(n=34)		Clinical(n=12)	
Biofilm grade*	No	%	No	%	No	%	No	%
Negative	7	31.8	2	4.8	28	82.4	1	8.3
Weak	15	68.2	26	61.9	6	17.6	7	58.3
Moderate	0	0	13	30.9	0	0	3	25
Strong	0	0	1	2.4	0	0	1	8.3

* The capacity for biofilm formation was scored as described by Satake *et al*. [16].

among clinical isolates with percentage of (16.7%) (Revise with Table 4). Nearly similar findings were reported by Cariolato *et al.* [8] and Gomes *et al.* [23].

As gelatinase activity was also phenotypically evaluated in an agar medium containing gelatin. It is interesting to note that many isolates carrying the *gel* gene were unable to degrade gelatin, suggesting either the existence of silent genes or that *gel* gene expression depends on environmental and culture conditions, that highlights the need of phenotypic and genotypic approaches to define the safety of enterococci strains [6, 13, 23, 24].

On the other hand, the gene *hyl* was not detected in both species analyzed from dairy origin, while it was detected in both species of clinical origin with percentage ranging from 69.1 to 41.6 %. Nearly similar findings were reported by Gomes *et al.* [23].

None of the E. faecium strains and 7(31.8%) of *E. faecalis* strains from dairy products produced *ESP*. While both strains from clinical samples produced ESP with percentage of 75 and 95.2, respectively (Table 4). Nearly similar results for food isolates were reported by Eaton and Gasson [5] and Valezuela *et al*. [25].

The data suggest that the *ESP* gene plays a role in pathogenicity because of the higher number of positive clinical *E. faecalis* strains as *ESP* is supposed to prevent revise the primary attachment to biotic and abiotic surface and to be involved in hiding the protein from the immune system [14]. In addition to its role in adhesion or attachment to biotic and abiotic surface, *ESP* is also thought to play a role in evasion of the immune response [26]. Thus, enterococci possessing this trait would clearly be undesirable for use in foods as a starter culture [13].

A strong association between the presence of *ESP* and biofilm formation in *E. faecalis* has been reported by Toeldo-Arana *et al.* [14]. In the same study, it was suggested that the presence of *ESP* rather than the phenotype (adherence or biofilm formation) is a good marker for identification of strains that are highly adherent to abiotic surfaces. Our results confirm this association for both *E. faecalis* and *E. faecium* isolates tested. Nearly similar findings were reported by Dupre *et al.* [27].

Haemolysin production can increase the severity of enterococci infections and the presence of genes involved in haemolysin /cytolysin production is also considered a risk factor [28]. Some of the tested isolates produced cytolysin with incidence of this trait being higher for *E. faecalis* isolates (13.6 and 40.5%) than for *E. faecium* isolates (2.9 and 25 %) from dairy products and clinical origin, respectively.

The incidence of cytolysin in our study was much lower than that reported by Ike *et al.* [29] for clinical isolates. In addition, our result are lower than the 44% incidence for *E. faecalis* strains isolated from foods reported by Eaton and Gasson. [5].

As *A*SA is an important virulence factor for enterocooci, that seems to mediate the specific binding of enterococci to intestinal epithelium, renal cells, human neutrophils and macrophages. In our study, all 34 *E. faecium* isolates from dairy products were ASA-negative, where as 10 (45.5%) of the 22 *E. faecalis* isolates were positive. While higher incidence of *ASA* gene was detected in *E. faecalis* and *E. faecium* isolates from clinical samples with percentages of 66.6 and 41.6 %, respectively. The same figures were rported by Eaton and Gasson [5].

Biofilm formation by enterococci has been recently recognized as a factor that contributes for pathogenicity. In our study, none of the dairy products isolates presented moderate or strong ability to form biofilm on abiotic surfaces, but most *E. faecalis* (68.2%) showed a weak ability to form biofilm (Table 5). Similar findings were reported by Gomes *et al*. [23]

In contrast, moderate and strong biofilim ability were shown in strains of *E. feaclis* and *E. feacium* isolated from clinical samples with percentage of 30.9 and 8.3 %, respectively.

According to Toeldo-Arana *et al.* [14] and Di Rosa *et al.* [30], biofilm formation can be modulated by environmental conditions, gelatinase activity and presence of *ESP* gene. However, Mohamed and Murray [31] found no correlation between biofilm formation and gelatinase production in clinical isolates of enterococci.

The presence of *ESP* gene in higher incidence in both strains of clinical isolates (95.2 and 75%), might indicate the influence of this gene on biofilm formation as well as suggested that this gene may represent an important factor to determine safety aspects of enterococci.

In conclusion, when enterococcal strains are selected as starter cultures, for safety considerations each strain should be tested for the different virulence traits. Yet, the question wither enterococci are safe for use as starter cultures remains difficult to answer.

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