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Bacteriocinogenic potential and safety evaluation of non-starter *Enterococcus faecium* strains isolated from home made white brine cheese

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ABSTRACT

Four LAB strains, isolated from Bulgarian home made white brine cheese, were selected for their effective inhibition against *Listeria monocytogenes*. According to their biochemical and physiological characteristics, the strains were classified as members of *Enterococcus* genus, and then identified as *Enterococcus faecium* by 16S rDNA sequencing.

Their bacteriocin production and inhibitory spectrum were evaluated together with the occurrence of several bacteriocin genes (*ent*A, *ent*B, *ent*P, *ent*L50B). Their virulence potential and safety was assessed both using PCR targeted to the genes *gel*E, *hyl*, *asa*1, *esp*, *cyl*A, *efa*A, *ace*, *van*A, *van*B, *hdc*1, *hdc*2, *tdc* and *odc* and by phenotypical tests for antibiotic resistance, gelatinase, lipase, DNAse and α- and β-haemolysis. The *E. faecium* strains harboured at least one enterocin gene while the occurrence of virulence, antibiotic resistance and biogenic amines genes was limited.

Considering their strong antimicrobial activity against *L. monocytogenes* strains, the four *E. faecium* strains exhibited promising potential as bio-preservatives cultures for fermented food productions.

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1. Introduction

In the last few decades consumers increased their demand for natural and chemical additive-free products urging the food industry to look for novel and alternative strategies for food biopreservation (Javed et al., 2011; Balciunas et al., 2013). One of the proposed routes was the use of bacteriocins produced by lactic acid bacteria (LAB), defined as ribosomally synthesized antimicrobial peptides that exhibit antagonism mainly against Gram-positive bacteria (Cotter et al., 2005; Gillor et al., 2008). Their bactericidal mechanisms vary and may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rDNA and inhibition of peptidoglycan synthesis (Heu et al., 2001). At the present time, only nisin and pediocin PA-1 (as pure or semi-purified preparations) are commercially authorized worldwide depending on local law regulation.

Due to their tolerance to salts and acids, *Enterococcus* spp. strains are highly adapted to several food systems. They are often found in high numbers and are believed to contribute to cheese ripening and to the development of aroma, especially in cheese products made in the Mediterranean area (Foulquié-Moreno et al., 2006), due to proteolysis and lipolysis, and production of diacetyl (Giraffa, 2002). *Enterococcus* spp. species have been reported to produce bacteriocins belonging to different classes. Most of these bacteriocins are produced by *Enterococcus faecium* and *Enterococcus faecalis* (reviewed in Giraffa, 1995; Moreno et al., 2003).

Some enterococci have been also investigated with regard to their potential as probiotics (Giraffa, 1995; Foulquié-Moreno et al., 2006; Sparo et al., 2008; Todorov and Dicks, 2008). However, such a role is still controversial considering both their increased association with nosocomial infections and their harbouring multiple antibiotic-resistant genes, transmissible by conjugation to pathogenic microorganisms (Dicks et al., 2011; Montalban-Lopez et al., 2011). In addition, several putative virulence factors have been described in enterococci, such as aggregation substance protein, gelatinase, cytolysin, enterococcal surface proteins,

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hyaluronidase, accessory colonisation factors and endocarditis antigens (Barbosa et al., 2010; Vankerckhoven et al., 2004). On the other hand, few studies have recently demonstrated safe application of enterococci in foods (Giraffa, 2002).

In this study, non-starter bacteriocin-producing strains isolated from home made goat white brine cheese produced in Belogratchik (Bulgaria) were evaluated for their bacteriocinogenic potential as well as for their beneficial and technological properties. The strains have been identified to be *E. faecium*, the bacteriocins partially characterised and its mode of action studied. Moreover, their safety traits have been determined. To our knowledge, this is the first report on the production of bacteriocins by *E. faecium* isolated from goat white brine cheese from Bulgaria. The final objective of this work was to characterize the produced bacteriocins, with the future aim of using the strains as potential biopreservatives cultures in cheese/milk fermentations.

2. Materials and methods

2.1. 1. Isolation and identification of LAB

Bacteriocinogenic LAB were isolated from home made goat white brine cheese produced in Belogratchik, Bulgaria. 50 g of cheese were homogenized with 450 mL of saline solution [0.85% (w/v) NaCl] in a Stomacher (Laboratory Blender Stomacher 400, Seward, England). Serial decimal dilutions were prepared in physiological saline solution and aliquots plated on MRS agar (2%, w/v) plates (Difco, BD, Franklin Lakes, NJ, USA) and incubated for 48 h at 37 °C. LAB were enumerated and plates presenting 15–20 colonies were covered with 10 mL of BHI (Oxoid, Basingstoke, UK) containing 1% (w/v) agar and 10^6 CFU/mL of *Listeria monocytogenes* Scott A. After incubating the plates for 24 h at 37 °C, single colonies displaying an inhibition zone were selected, grown on MRS for purification and examined for colony morphology, production of catalase, acidification and microscopic characteristics (Gram staining and cell shape and size).

Catalase negative and Gram-positive isolates that produced acid were then tested for their ability to grow in skim milk at 10 °C and 45 °C, in MRS broth (Difco) at pH 4.4 and 9.6, and in the presence of NaCl 6.5% (w/v). These bacteria were screened for bacteriocin production by agar-spot-test method (Todorov and Dicks, 2005) against members of the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Listeria* and *Pediococcus* (Table 1). Activity was expressed as arbitrary units (AU) per mL, with one AU defined as the highest dilution showing a clear zone of inhibition (Todorov and Dicks, 2005). *L. monocytogenes* ATCC 7644, *L. monocytogenes* Scott A, *Listeria innocua* ATCC 33090 and *Listeria ivanovii subsp. ivanovii* ATCC 19119 were used as a sensitive test strain.

The bacteriocin-producing isolates were pre-identified using API50CHL and API20Strep system (BioMerieux, Marcy-l'Etoile, France). The DNA of the microorganisms was extracted with the ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA) and further evaluated by use of the random amplification of polymorphic DNA (RAPD) PCR technique with primers OPL-02 (5′-TGG GCG TCA A-3′), OPL-04 (5′-GAC TGC ACA C-3′), OPL-14 (5′-GTG ACA GGC T-3′) and OPL-20 (5′-TGG TGG ACC A-3′) (Todorov et al., 2010). The amplified products were separated by electrophoresis in 1.4% (w/v) agarose gels in TAE buffer at 100 V for 2 h. Gels were stained in TAE buffer containing 0.5 μg/mL ethidium bromide (Sigma–Aldrich Diagnostics, St. Louis, MO, USA).

Based on results of RAPD-PCR and size of the inhibition zone, isolates ST209GB, ST278GB, ST315GB and ST711GB were selected for future studies. The microorganisms were identified by the use of PCR genus-specific primers Ent1 and Ent2 (Todorov et al., 2010) and further confirmed by amplification of 16S rDNA with primers F8 and R1512 (Felske et al., 1997). Amplification products were checked by agarose gel electrophoresis, purified (QIAquick PCR Purification Kit – Qiagen, Hilden, Germany) and then subjected to sequencing. Species identification was performed after BlastN alignment (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the obtained sequences with those present in the GenBank public database. A minimum sequence similarity level of 99% was considered for species identification.

2.2. Characterization of the bacteriocins

2.2.1. Isolation of bacteriocins

Strains ST209GB, ST278GB, ST315GB and ST711GB were cultured in MRS broth for 24 h at 37 °C. The cells were harvested (8000 × *g* for 10 min at 4 °C), the cell-free supernatant was adjusted to pH 6.5 with 1 M NaOH, heat-treated (80 °C for 10 min) and the amount of

Table 1
Spectrum of antibacterial activity of bacteriocins ST209GB, ST278GB, ST315GB and ST711GB produced by *Enterococcus faecium* strains.

Test microorganisms	Medium	Incubation temperature (°C)	ST209GB	ST278GB	ST315GB	ST711GB
Enterococcus faecalis	MRS	30	6/6[a]	6/6	6/6	6/6
Enterococcus faecium	MRS	30	4/9	4/9	3/9	3/9
Enterococcus mundtii	MRS	30	1/1	1/1	1/1	1/1
Enterococcus spp.	MRS	30	16/21	19/21	18/21	19/21
Lactobacillus acidophilus	MRS	30	0/2	0/2	0/2	0/2
Lactobacillus curvatus	MRS	30	0/3	0/3	0/3	0/3
Lactobacillus delbrueckii	MRS	30	0/2	0/2	0/2	0/2
Lactobacillus fermentum	MRS	30	0/3	0/3	0/3	0/3
Lactobacillus paracasei	MRS	30	6/8	6/8	5/8	5/8
Lactobacillus plantarum	MRS	30	0/7	0/7	0/7	0/7
Lactobacillus sakei	MRS	30	0/5	0/5	0/5	0/5
Lactococcus lactis	MRS	30	0/3	1/3	2/3	0/3
Leuconostoc mesenteroides subsp. *mesenteroides*	MRS	30	0/4	0/4	0/4	0/4
Listeria innocua	BHI	37	2/3	2/3	2/3	3/3
Listeria monocytogenes	BHI	37	24/26	23/26	26/26	22/26
Listeria ivanovii subsp. *ivanovii*	BHI	37	2/2	2/2	2/2	2/2
Pediococcus spp.	MRS	30	0/4	0/4	0/4	0/4
Salmonella spp.	BHI	37	0/7	0/7	0/7	0/7
Clostridium spp.	BHI	37	0/4	0/4	0/4	0/4
Escherichia coli	BHI	37	0/6	0/6	0/6	0/6

[a] Number of strains inhibited out of the total number of tested strains.

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