Influence of Enterococci and Lactobacilli on *Listeria*

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Abstract: A collection of lactic acid bacteria (38 *Enterococcus* and 41 *Lactobacillus* strains) was tested for the antilisterial activity against 15 *Listeria* spp. strains (two *L. monocytogenes*, one *L. ivanovii* and 12 *L. innocua* strains) using agar spot method. Out of all 79 bacteria only six *Enterococcus* strains (1/3A, 3/3A, 6/4D, 6/1A, 1282 and EN3) exhibited antilisterial activity against almost all used indicator strains, when their live cells were tested. When their cell free neutralised supernatants (CFNS) were tested against four selected indicator strains (*L. innocua* Ln-03, Ln-06, Ln-10 and *L. monocytogenes* CCM5576) only two *Enterococcus* spp. strains were active – *E. faecalis* 6/1A strain from raw cow milk of minor interest due to the activity of its CFNS only against *L. innocua* Ln-06 and thermolability of the compound and *E. mundtii* 1282 strain from goat raw milk with CFNS active against 13 *Listeria* spp. strains including *L. monocytogenes*. *E. mundtii* 1282 strain produced probably a bacteriocin, because it completely lost the activity after treatment CFNS with proteinase K.

Keywords: *Enterococcus*; *Lactobacillus*; *Listeria*; antilisterial activity

Listeria monocytogenes is the causative agent of human listeriosis, a potentially fatal foodborne infection. Clinical manifestations range from febrile gastroenteritis to more severe invasive forms including meningitis, encephalitis, abortions, and perinatal infections. This Gram-positive facultative intracellular pathogen has evolved multiple strategies to face extracellular innate defense mechanisms of the host and to invade and multiply intracellularly within macrophages and nonphagocytic cells (Dussurget 2008).

Several outbreaks of listeriosis associated with the consumption of milk and dairy products have occurred since 1980 and are causing great concern to the dairy industry, owing to the number of cases and the nearly 30% overall mortality rate of these outbreaks. Initiatives undertaken by the industry in response to the threat of contamination of milk and dairy products by *L. monocytogenes* have in general led to a more satisfactory control of the pathogen. *L. monocytogenes* may directly contaminate milk as a consequence of listerial mastitis, encephalitis or *Listeria*-related abortion in cattle and asymptomatic cows can also shed *L. monocytogenes* in their milk for many months. Under unhygienic milking practices indirect contamination of bulk milk is likely to occur if *L. monocytogenes* is present in feeds, faeces, udder surface or bedding. *L. monocytogenes* survives during manufacture and ripening of most cheese varieties, and is likely to grow in cheese if the pH reaches higher values (Gaya *et al.* 1998).

As a consequence of listeriosis outbreaks, with soft cheese being the major food vehicle, today it is well established that this type of cheese is amongst the products that pose the highest risk with regard to human listeriosis. Concern over cheese-borne listeriosis has prompted research investigations to examine the use of naturally occurring microflora, such as bacteriocin-producing lactic acid bacteria,

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to reduce the incidence of *L. monocytogenes*. Most studies on soft cheese have used bacteriocin-producing bacteria as starter cultures in milk and focused principally on white-mold cheeses such as Camembert (Izquierdo *et al.* 2009). However, most probably due to post-process smearing operations, the incidence of *L. monocytogenes* in red smear soft cheeses seems to be higher than in mold-ripened cheeses (Rudolf & Scherer 2001; Izquiedro *et al.* 2009). In fact, smear cheeses are regularly washed with a brine solution, which represents a major means of contamination and cross-contamination with *L. monocytogenes*. In addition, these cheeses provide excellent growth conditions particularly on the surface where a rising pH-gradient develops during ripening creating a more suitable environment for the growth of *L. monocytogenes*. Interestingly, the smearing operation can also be used as a means of combating the pathogens by adding a bacteriocin producer to the brine. This approach, which was developed in a previous report on the use of the pediocin AcH producing *Lactobacillus plantarum* WHE92 as a surface culture, proved to be a viable strategy in helping to reduce cheese contamination with *Listeria*. These results are mainly due to the fact that *L. monocytogenes* is almost exclusively localised on the surface of smear cheeses. In recent years, enterococci and lactobacilli were the focus of numerous investigations on bacteriocin production, mainly because bacteriocin production seems to be a common trait among strains associated with food systems. A large number of reports are available on bacteriocin-producing *Enterococcus* and *Lactobacillus* strains. Such strains are in fact easily isolated from various foods and are most likely to play a role in influencing the content of *L. monocytogenes* in food matrices, especially since a vast majority of enterocins (enterococcal bacteriocins) are active against this pathogen of great concern to public health (Izquierdo *et al.* 2009) and many bacteriocins produced by lactobacilli are active as well (Martinez & De Martinis 2005; Ghalfi *et al.* 2006; Zhou *et al.* 2008).

The aim of this study was to determine antilisterial activity of enterococci and lactobacilli, which can create the main part of NSLAB of different cheese including the smear cheese, against *Listeria* spp. in respect of the possibility to use them in the future as a part of microflora with protective properties in smear cheese production.

MATERIALS AND METHODS

Microorganisms and media. All used microorganisms are summarised in Table 1 and were cultivated at 37°C for 18 h aerobically (lactobacilli were cultivated anaerobically). *Listeria* spp. were cultivated in BHI broth (Himedia), lactobacilli and enterococci in MRS broth (Oxoid).

Preparation of culture supernatant. Strains with antilisterial activity were grown according to their optimal conditions of cultivation. The cultures were centrifuged at 3680 g for 15 min at 4°C, the cell-free supernatant was pH neutralised to pH 6.0–6.5 using NaOH (100 g/l solution) and heated at 90°C for 10 min to inactivate the remaining cells. The cell-free, neutralised supernatant (CFNS) was used in further bacteriocin characterisation experiments (Franz *et al.* 1996).

Screening of antilisterial activity. First, the antibacterial activity of the live cells was tested using 10 µl volumes of the 18-h culture from the MRS broth. The volumes (10 µl) were spotted onto the surface of an BHI soft (7 g/l) agar (7 ml, Himedia), which had been inoculated with 70 µl of an overnight culture of the indicator strain diluted to the final concentration of 10^5–10^6 CFU/ml. The assay plates were incubated at 37°C for 24 hours. When some activity was observed, the CFNS was tested using an agar spot test as described above (Schillinger *et al.* 1993).

Effect of enzymes on antilisterial activity. The CFNS was treated with enzymes: catalase (2860 U/mg, Sigma Aldrich, USA) and proteinase K (52 U/mg, Sigma Aldrich, USA). Each enzyme was dissolved in sterile demineralised water and added to the CFNS to the final concentration of 1.0 mg/ml. Following incubation, at 37°C for 2 h, the reaction mixtures were heated to 100°C for 10 min to inactivate the enzymes before assessing the remaining bacteriocin activity against selected indicator strains (Kang & Lee 2005).

RESULTS AND DISCUSSION

For the detection of the antilisterial activity a collection of 15 *Listeria* strains (two *L. monocytogenes* strains, one *L. ivanovii* strain and 12 *L. innocua* strains) as indicator strains and 38 *Enterococcus* and 41 *Lactobacillus* strains as tested strains was selected. First, the live cells were tested for antilisterial activity using agar spot test against

Table 1. Origin of selected microorganisms

Strain	Source
Listeria spp.	
L. monocytogenes NCTC4886	National Collection of Type Cultures, UK
L. monocytogenes CCM5576	Czechoslovak Collection of Microorganisms, Brno, CR
L. innocua Ln-01, Ln-02, Ln-03, Ln-04, Ln-06, Ln-08, Ln-09, Ln-10, Ln-11, Ln-12, Ln-13	DBM, ICT Prague, CR
L. innocua CCM4030	Czechoslovak Collection of Microorganisms, Brno, CR
L. ivanovii CCM5884	Czechoslovak Collection of Microorganisms, Brno, CR
Enterococcus spp.	
E. mundtii 22, 36, 1282, 1317, 1333, 1342, 1346, 1400, 1422, 1439, 1569	University of Veterinary and Pharmaceutical Sciences, Brno, CR
E. mundtii CCM4059	Czechoslovak Collection of Microorganisms, Brno, CR
E. mundtii EN3, EN14, EN15	DDFT, ICT Prague, CR
E. faecalis 1/2B, 1/2D, 1/3A, 1/3C, 2/1B, 3/1B, 3/2B, 3/2E, 3/3A, 3/3C, 4/1A, 4/2C, 4/2D, 4/3C, 4/3D, 6/1A, 6/1B	DDFT, ICT Prague, CR
Enterococcus spp. 1/1A, 1/1B, 2/1A, 3/1A, 4/1B, 6/4D	DDFT, ICT Prague, CR
Lactobacillus spp.	
Lbc. acidophilus CH5	Christian Hansen's Laboratory, Denmark
Lbc. fermentum ST61	DDFT, ICT Prague, CR
Lbc. rhamnosus 65, 81, 85, 91, 123, 161, 163, 173, 183, 202	STU, Bratislava, SR
Lbc. rhamnosus VT1, LBK7, DMF30129, NK10, NK20	DDFT, ICT Prague, CR
Lbc. paracasei 01, 02, 05, SF1, 011	DFST, University of Nebrasca, USA
Lbc. paracasei 171R2, 7R1, 8R2, 171M7, 61H4	KVL, Denmark
Lbc. paracasei ST68, ST491	DDFT, ICT Prague, CR
Lbc. plantarum LHI10, NK30	DDFT, ICT Prague, CR
Lbc. plantarum NCDO1752	National Collection of Dairy Organisms, UK
Lbc. casei 2750, Shirota	CFRI, Budapest, Hungary
Lbc. casei NCDO161	National Collection of Dairy Organisms, UK
Lbc. casei 148, 154, 158-1, 150-1, 150-2	Milcom, Prague, CR
Lbc. casei CH1	Christian Hansen's Laboratory, Denmark

the indicator strains and when some activity was observed, the CFNS of these strains were tested as well. *L. innocua* Ln-03, Ln-06, Ln-10 and *L. monocytogenes* CCM5576 were the most sensitive strains so these strains were used for other experiments. Only six *Enterococcus* strains (1/3A, 3/3A, 6/4D, 6/1A, 1282 and EN3) out of 79 enterococci and lactobacilli strains exhibited antilisterial activity against almost all used indicator strains, when their live cells were used. When their CFNS were tested against the selected indicator strains (*L. innocua* Ln-03, Ln-06, Ln-10 and *L. monocytogenes* CCM5576) only two *Enterococcus* spp. strains were active. This can be explained by an acid effect or by a presence of thermolable compounds (Ouwehand 1993).

The strain *E. mundtii* 1282 was isolated from raw goat milk and showed the strongest antilisterial activity (Figure 1). The second strain with active CFNS was strain *E. faecalis* 6/1A from raw cow milk and its CFNS was active only against *L. innocua* Ln-06 (Figure 2). In Table 2 there is summarised the effect of proteinase K and catalase on their antilisterial activity. The antilisterial activity of

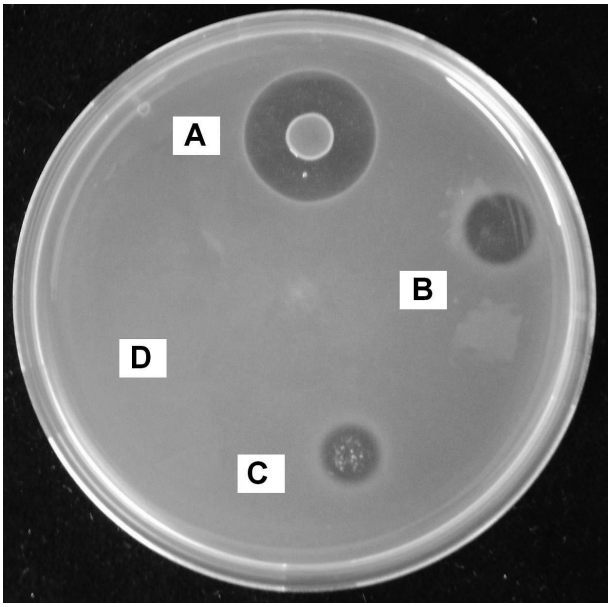

Figure 1. Effect of catalase and proteinase K on the antilisterial activity of *E. mundtii* 1282 strain against *L. monocytogenes* CCM5576: A – live cells; B – CFNS; C – CFNS + catalase; D – CFNS + proteinase K

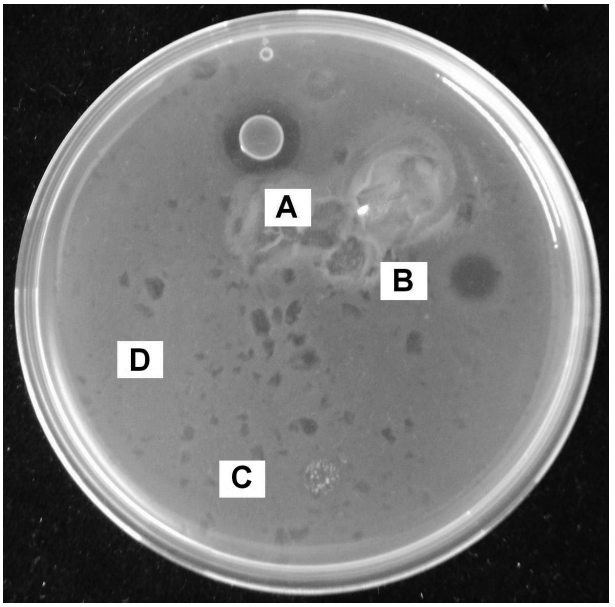

Figure 2. Effect of catalase and proteinase K on the antilisterial activity of *E. faecalis* 6/1A strain against *L. innocua* Ln-06: A – live cells; B – CFNS; C – CFNS + catalase; D – CFNS + proteinase K

E. faecalis 6/1A strain is caused not only by the production of acid but the strain probably produces a thermolable compound, because the activity was observed in one case for its CFNS. Due to this thermolability and the narrow spectrum of its activity of antilisterial compound this strain lost its importance. More significant results were obtained for *E. mundtii* 1282 strain. The substantial activity of its live cells was observed against 13 out of 15 indicator *Listeria* strains (except for *L. monocytogenes* NCTC4886 and *L. ivanovii* CCM5884) and slight reduction of activity was determined for its CFNS and CFNS after treatment with catalase when tested against selected four *Listeria* indicator strains. A complete loss of activity was caused after treatment of CFNS with proteinase K. These results suggest that strain *E. mundtii* 1282 produces probably a bacteriocin, because proteinaceous nature of its antilisterial compound was proved after proteinase K treatment. The antagonistic activity of its CFNS was not inhibited by catalase, which indicated that the inhibition observed was not due to hydrogen peroxide.

The antilisterial activity of enterococci is well known and can be explained by a close phylogenetic relationship between *Enterococcus* spp. and *Listeria* spp. (Moreno *et al.* 2006). The growth of *L. monocytogenes* was inhibited by enterocin SE-K4 (Eguchi *et al.* 2001), enterocins L50A and L50B (Cintas *et al.* 2000), enterocin AS-48RJ (Abriouel *et al.* 2005), entrocins A and B (Casaus *et al.* 1997), *E. casseliflavus* IM46KI (Sabia *et al*. 2002) and *E. faecium* EK13 (Mareková *et al.* 2003). *E. faecium* JBL1061, JBL1083 and JBL1351

Table 2. Effect of proteinase K and catalase on the antilisterial activity of *E. mundtii* 1282 and *E. faecalis* 6/1A strains

Indicator strain	Diameter of inhibition zone (mm)							
	Live cells		CFNS		CFNS + catalase		CFNS + proteinase K	
	1282	6/1A	1282	6/1A	1282	6/1A	1282	6/1A
L. innocua Ln-03	20.0	10.5	10.5	0	9.0	0	0	0
L. innocua Ln-06	20.3	12.0	14.0	6.0	12.0	0	0	0
L. innocua Ln-10	20.5	11.0	15.0	0	13.0	0	0	0
L. monocytogenes CCM5576	20.7	14.0	10.5	0	9.0	0	0	0

inhibited eight strains of *L. monocytogenes* out of nine strains (Arihara *et al.* 1993). Bacteriocin RC714 inhibited the growth of *L. monocytogenes, L. innocua, L. murrayi* and *L. grayi* (Del Campo *et al.* 2001). Enterocin P was also active against *L. monocytogenes* and *L. innocua* (Cintas *et al.* 1997). Enterocin EJ97 inhibited all six *Listeria* species (Gálvez *et al.* 1998).

None tested *Lactobacillus* strain showed any sign of an inhibition, although some of them possessed antibacterial and/or antifungal activity (Giesová *et al*. 2004; Plocková *et al*. 2004; Hudáček *et al*. 2007; Tůma *et al*. 2007, 2008). The ability of different lactobacilli to possess antilisterial activity was previously published as well. *Lbc. bavaricus* MI401 inhibited *L. monocytogenes* (Larsen & Norrung 1993), *Lbc. sakei* L45 inhibited *L. monocytogenes* and *L. innocua* (Chen & Hoover 2003). *Lbc. curvatus* LTH1174 (Ticha-czek *et al.* 1993), *Lbc. sakei* Lb706 (Holck *et al.* 1992), *Lbc. sakei* Lb674 (Hünke *et al*. 1996) and *Lbc. sakei* 2512 (Simon *et al.* 2002) inhibited *L. monocytogenes, L. ivanovii* and *L. innocua*.

CONCLUSIONS

Antilisterial activity of 38 *Enterococcus* and 41 *Lactobacillus* strains against 15 *Listeria* spp. strains was tested using agar spot method. Only *E. mundtii* 1282 strain from goat raw milk was active against 13 *Listeria* spp. strains including *L. monocytogenes*. This strain produced probably a bacteriocin, because the activity lost after treatment CFNS with proteinase K. This compound will be in the future characterise in detail (its tolerance to pH, heat and NaCl; its stability during storage), purified and used in laboratory conditions for studying its protective effect in smear-cheese model system.

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