

Antilisterial Activity of Lactic Acid Bacteria against *Listeria monocytogenes* Strains Originating from Different Sources

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Abstract:

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Eight individual bacteriocin-producing lactic acid bacteria (LAB) strains and three bacteriocin-non-producing cheese starter cultures were evaluated for their ability to inhibit the growth of six *Listeria monocytogenes* strains, originating from the guinea-pig lymph nodes, raw cow milk, and manufacturing dairy equipment. Results showed that either live cells or cell-free neutralised supernatant (CFNS) and/or heated CFNS of six individual LAB strains (*Lcc. lactis* subsp. *lactis* CCDM 416 and NIZO R5, *Lbc. plantarum* HV 11 and DC 1246, *P. acidilactici* HV 12, and *Ent. mundtii* CCM 1282) and one starter culture (DELVO-ADD® 100-X DSF) were effective in the suppression of at least one listeria strain. Neither any individual LAB strain nor starter culture was antagonistic toward all studied *L. monocytogenes* strains, indicating diverse sensitivity/resistance among *L. monocytogenes* strains to antimicrobial compounds of LAB. The significant susceptibility of listerias isolated from raw milk and dairy equipment together with the strong antilisterial activity of DELVO-ADD® 100-X DSF could be applied in dairy technology, where commonly used starter cultures could play both the biopreservative and fermentation role.

Keywords: *Listeria*; starter culture; antilisterial effect; bacteriocin; sensitivity; biopreservative agent

The ability of *Listeria monocytogenes* to survive in a wide range of unsuitable conditions, including relatively low pH (O'DRISCOLL *et al.* 1996), refrigerated temperatures (WALKER *et al.* 1990) and high concentrations of NaCl (FARBER & PETERKIN 1991) makes this foodborne pathogen particularly difficult to control.

A possible approach to naturally improve food safety and quality and to extend the shelf-life of foods is to search for those lactic acid bacteria (LAB) that would be able to suppress *L. monocytogenes* growth and, at the same time, would be inherent to food products. Starter cultures, containing specific combinations of LAB strains, play an essential role in the majority of food fermentations (ABEE *et al.* 1994). Moreover, LAB are well known as producers of

various antimicrobial metabolites, including organic acids, diacetyl, acetoin, ethanol, hydrogen peroxide, carbon dioxide, exopolysaccharides, enzymes, reuterin and bacteriocins – small proteins possessing direct activity towards closely related Gram-positive bacteria, including *L. monocytogenes* (DE VUGST & VANDAMME 1994). Although bacteriocins may be found in many bacteria, those produced by LAB as so-called GRAS (Generally Recognized as Safe) have received particular attention in recent years due to their potential application in the food industry as natural preservatives (ENNAHAR *et al.* 1999). The addition of bacteriocin-producing (Bac⁺) LAB or purified bacteriocins in order to protect foodstuffs has been studied (MURRAY & RICHARD 1997; CLEVE-

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LAND *et al.* 2001). The class I bacteriocin nisin, which is commercially available as a food preservative, and bacteriocins of class IIa (pediocin-like) are of special interest as inhibitors of *L. monocytogenes* (ENNAHAR *et al.* 1999). Most bacteriocins are able to kill target cells by the permeabilisation of cell membrane. It is well known that the antimicrobial activity of bacteriocins is very specific, since they employ specific receptors such as lipid II or mannose phosphotransferase system on the sensitive target cell surfaces (GRAVESEN *et al.* 2002). Sensitivity to both nisin and pediocin-like substance among *L. monocytogenes* strains is divergent and designated as strain-dependent (UKUKU & SHELEF 1997). The exact knowledge of the susceptibility of target microorganism is necessary in order to use bacteriocins in the food protection. Intrinsic properties of food-stuffs could also significantly influence the effect of bacteriocins (LIU & HANSEN 1990). The big concern in this area is the danger of development of highly

tolerant and/or resistant strains. It has been observed that listerias develop tolerance towards nisin and pediocin-like substance (PLS) in laboratory media at relatively high frequency (REKHIF *et al.* 1994). In addition, the resistance to a bacteriocin may extend to other bacteriocins within the same class or even in other classes and thus create multi-resistance listeria strains (NAGHMOUCHI *et al.* 2007).

The aim of this study was to screen the set of eight individual Bac⁺ LAB strains and three Bac[−] commercial cheese starter cultures for their ability to suppress the growth of six *L. monocytogenes* strains, originating from different sources, in order to assess their sensitivity to antimicrobial compounds produced by LAB.

MATERIAL AND METHODS

Bacterial strains. All used *L. monocytogenes* strains, individual LAB strains and cheese starter cultures are summarised in Table 1.

Table 1. Origin and characteristics of used bacteria

Strain	Characteristic	Source
<i>L. monocytogenes</i> CCM 5576	Collection strain, originating from guinea-pig lymph nodes, serovar 1/2a	CCM Brno, CZ
<i>L. monocytogenes</i> L-2296 (Lm-25)	Isolate from raw cow milk (storage tank), serovar 1/2a	DBM, ICT Prague, CZ
<i>L. monocytogenes</i> L-2299 (Lm-26)	Isolate from raw cow milk (cistern), serovar 1/2a	DBM, ICT Prague, CZ
<i>L. monocytogenes</i> L-2300 (Lm-29)	Isolate from raw cow milk, serovar 1/2a	DBM, ICT Prague, CZ
<i>L. monocytogenes</i> L-2297 (Lm-30)	Isolate from raw cow milk, serovar 1/2a	DBM, ICT Prague, CZ
<i>L. monocytogenes</i> Lm-31*	Isolate from the surface of manufacturing dairy equipment	DBM, ICT Prague, CZ
<i>Lcc. lactis</i> subsp. <i>lactis</i> CCDM 416	Lowly lytic strain, strong fermentation ability, production of nisin A (ŠVIRÁKOVÁ <i>et al.</i> 2009)	Laktoflora [®] , MILCOM a.s., CZ
<i>Lcc. lactis</i> subsp. <i>lactis</i> NIZO R5	Lytic strain, production of nisin A (RAUCH <i>et al.</i> 1992)	Netherlands Institute for Dairy Research, NL
<i>Lcc. lactis</i> subsp. <i>lactis</i> CCDM 731	Isolate from mountain forests, strong fermentation ability, production of nisin A	Laktoflora, MILCOM a.s., CZ
<i>Lcc. lactis</i> subsp. <i>lactis</i> LTM 32	Isolate from Vietnamese fermented milk, production of bacteriocin (Do <i>et al.</i> 2001)	DDFC, ICT Prague, CZ
<i>Lbc. plantarum</i> HV 11	Isolate from the French cheese Ami du Chambertin, production of bacteriocin (VLKOVÁ & PLOCKOVÁ 2011)	DDFC, ICT Prague, CZ
<i>Lbc. plantarum</i> DC 1246	Collection strain, production of bacteriocin	Danisco, D
<i>P. acidilactici</i> HV 12	Isolate from soft cheese, production of bacteriocin (VLKOVÁ & PLOCKOVÁ 2011)	DDFC, ICT Prague, CZ
<i>Ent. mundtii</i> CCM 1282	Isolate from goat milk, production of bacteriocin (SOLICHOVÁ <i>et al.</i> 2012)	VFU Brno, CZ
DELVO-ADD [®] 100-X DSF	<i>Lcc. lactis</i> subsp. <i>lactis</i> , <i>Lcc. lactis</i> subsp. <i>cremoris</i> , <i>Lcc. lactis</i> subsp. <i>lactis</i> biovar. <i>diacetylactis</i> , <i>Leuconostoc</i> sp.; nisin-non-producing (ŠVIRÁKOVÁ <i>et al.</i> 2009)	O.K. SERVIS BioPro, CZ
DELVO-UX [®] 11B DSF		
DELVO-TEC [®] LL 50A-Z DSF	<i>Lcc. lactis</i> subsp. <i>lactis</i> , <i>Lcc. lactis</i> subsp. <i>cremoris</i> ; resistance to phages, nisin-non-producing (ŠVIRÁKOVÁ <i>et al.</i> 2009)	

*serovar not determined yet; DBM – Department of Biochemistry and Microbiology; DDFC – Department of Dairy and Fat, and Cosmetics; ICT – Institute of Chemical Technology; VFU – University of Veterinary and Pharmaceutical Sciences

Cultivation conditions of bacteria. Individual listeria, enterococci and pediococci strains were cultivated in BHI broth (HiMedia, Mumbai, India) at 30°C for 18 h, aerobically. Lactococci and cheese starter cultures were cultivated in M17 broth (Oxoid, Basingstoke, UK) at 30°C for 18 h, aerobically. Lactobacilli were cultivated in MRS broth (Oxoid, Basingstoke, UK) at 37°C for 18 h, aerobically.

Preparation of cell-free, neutralised and heated supernatants. Individual LAB strains and cheese starter cultures were centrifuged at 3680 g for 15 min at 4°C. The cell-free supernatants (CFS) were neutralised to pH 6.5 using NaOH solution (100 g/l solution). Neutralized cell-free supernatants (CFNS) were heated at 90°C for 10 min to inactivate remaining bacterial cells, enzymes and thermolabile compounds (FRANZ *et al.* 1996). For each experiment, CFS, CFNS and heated CFNS were always prepared fresh.

Screening of antilisterial activity. Antilisterial activities were tested by the agar diffusion method. BHI soft agar (7.5 g/l, 10 ml) (HiMedia, Mumbai, India) was inoculated (inoculum 1% v/v) with freshly grown *L. monocytogenes* strain (10^9 CFU/ml) and poured on Petri dish. After solidification and drying up, freshly grown cells of individual LAB strains and cheese starter cultures (10^9 CFU/ml), their CFNS and heated CFNS were spotted (10 µl) on the surfaces of BHI soft agar. The assay plates were incubated at 30°C for 20 h, aerobically (SCHILLINGER *et al.* 1993). After that, the plates were checked for the presence of clear growth inhibition zones around the spots in order to determine the antilisterial activity of fresh cells, CFNS and heated CFNS of the used individual LAB strains and cheese starter cultures. The diameters of inhibition zones were measured. Results represent an average of three realized experiments.

RESULTS AND DISCUSSION

The used eight individual Bac⁺ LAB strains (*Lcc. lactis* subsp. *lactis* CCDM 416, NIZO R5, CCDM 731 and LTM 32, *Lbc. plantarum* HV 11 and DC 1246, *P. acidilactici* HV 12, and *Ent. mundtii* CCM 1282) were chosen for this study because of their significant potential for the application in fermented dairy technology as biopreservative agents. The antilisterial activity of these strains against non-pathogenic *L. innocua*, commonly used as indicator strain, was previously confirmed (KUČEROVÁ *et al.* 2009; ŠVIRÁKOVÁ *et al.* 2009). The used three nisin-non-producing (Nis⁻) cheese starter cultures (DELVO-TEC[®] LL

50A-Z DSF, DELVO-ADD[®] 100-X DSF and DELVO-UX[®] 11B DSF) did not previously demonstrate any antilisterial activity (ŠVIRÁKOVÁ *et al.* 2009), nevertheless, because of their common application in cheese technology, their antilisterial potential was also evaluated. One collection strain from guinea-pig lymph nodes (*L. monocytogenes* CCM 5576) and six isolates from raw cow milk (*L. monocytogenes* Lm-25, Lm-26, Lm-29, and Lm-30) and manufacturing dairy equipment (*L. monocytogenes* Lm-31) were tested as target strains. Observed results are summarised in Figure 1. This study confirmed that six individual LAB strains and one starter culture were able to inhibit at least one *L. monocytogenes* strain. However, neither any individual LAB strain nor starter culture was able to inhibit all studied listerias. Our results indicated different sensitivity among *L. monocytogenes* strains to antilisterial compounds produced by LAB, including mainly organic acids and thermolabile and/or thermostable compounds, probably bacteriocin(s).

Figure 1 shows that live cells and CFNS of two Nis⁺ lactococci (*Lcc. lactis* subsp. *lactis* CCDM 416 and NIZO R5) were effective especially in the suppression of collection strain *L. monocytogenes* CCM 5576. No significant decrease of antilisterial activity caused by CFNS in comparison with live cells confirmed that organic acids were not the most effective inhibitory substances. Conversely, the antilisterial activity of heated CFNS was not observed. According to this, nisin, possessing the highest activity in acidic pH (LIU & HANSEN 1990) and known as thermostable (HURST 1981), was not confirmed as the main antilisterial agent for CCDM 416, as was primarily assumed. Probably, the production of another unspecified thermolabile compound is expected. Conversely, the antimicrobial potential of both tested lactococci was not effective in the inhibition of other tested listerias (Figures 1b–f), isolated from raw milk and dairy equipment. It could be explained by varying degrees of sensitivity to antimicrobial compounds observed in *L. monocytogenes* (UKUKU & SHELEF 1997). It is known that nisin is able to form pores in the cytoplasmatic membrane resulting in the cell death (MOLL *et al.* 1996). However, the activity of nisin depends on various other factors such as energized membranes of susceptible microorganisms, concentration of nisin and concentration of inhibited cells (GAO *et al.* 1991; WILLIAMS & DELVES-BROUGHTON 2003). Moreover, some listerias could become immune probably through the outgrowth of a spontaneous mutant population resistant to nisin (HANLIN *et al.*

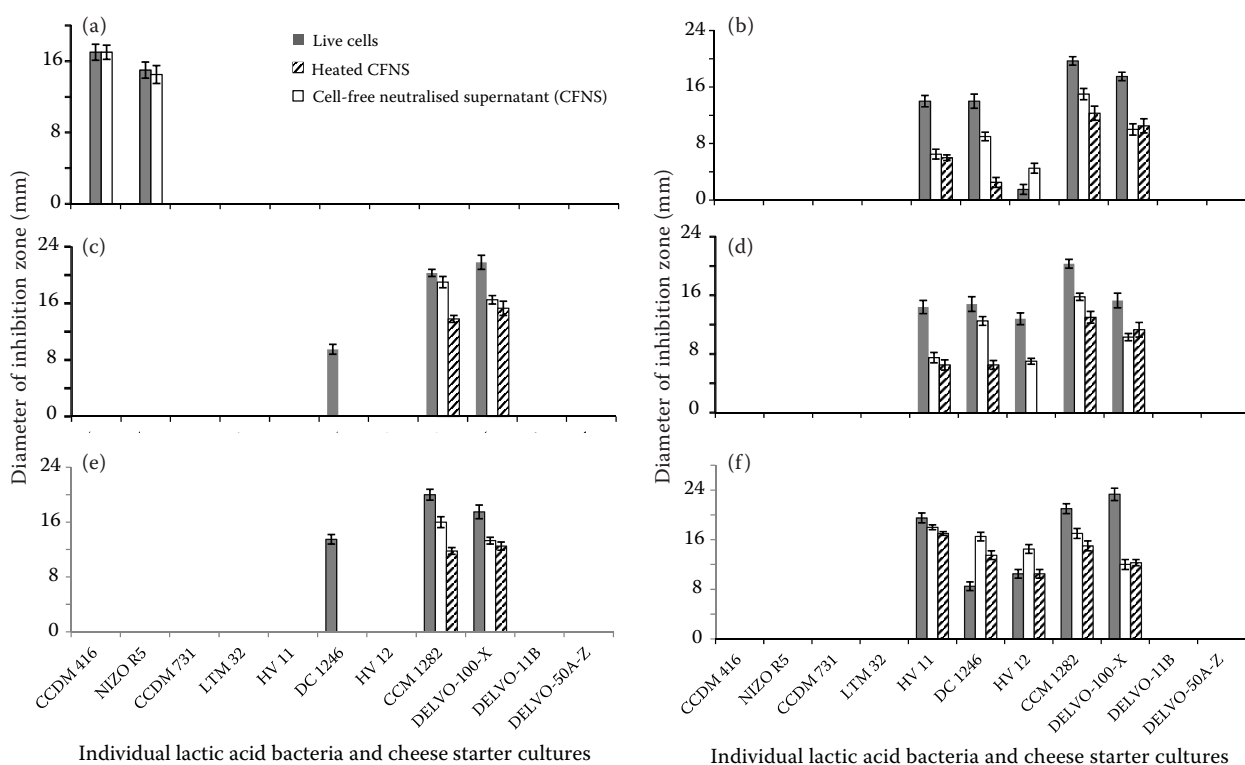


Figure 1. Antilisterial activity of individual LAB strains and cheese starter cultures against *L. monocytogenes* CCM 5576 (a), *L. monocytogenes* Lm-25 (b), *L. monocytogenes* Lm-26 (c), *L. monocytogenes* Lm-29 (d), *L. monocytogenes* Lm-30 (e), and *L. monocytogenes* Lm-31 (f)

1993). Although the majority of the initial target cells are inhibited by nisin, some cells can escape and regrow after an apparent lag time (REKHIF *et al.* 1994). The mechanism of nisin resistance is attributed to changes in the cell membrane (fatty acid composition and fluidity) and S-layer (MANTOVANI & RUSSELL 2001). In addition, it seemed that listerias that were shown to be resistant to lactococci could be resistant to both nisin and undefined thermolabile compound, and possessed significant antilisterial activity against *L. monocytogenes* CCM 5576. From this aspect, it is possible that nisin resistance could confer cross-resistance to other chemically and structurally different antimicrobial compounds (MAZZOTA & MONTVILLE 1997). It was observed that the origin of listerias could play an important role in their sensitivity to LAB. However, the resistance of three of 245 *L. monocytogenes* isolates from a variety of origins (human case of listeriosis, smoked salmon and pig faeces) to bacteriocin bavaricin A did not correlate with the strain origin (LARSEN & NORRUNG 1993). MARTINEZ *et al.* (2005) reported that nisin-resistant variants of *L. monocytogenes* are able to grow in milk fermented by Nis⁺ lactococci, although the growth of the wild types of *L. monocytogenes*

strains is inhibited. It indicates the unpredictability of nisin resistance development in a dairy environment (MARTINEZ *et al.* 2005).

Figures 1b–f show that both Bac⁺ lactobacilli (*Lbc. plantarum* HV 11 and DC 1246) were effective in the suppression of three *L. monocytogenes* strains (Lm-25, Lm-26 and Lm-31), namely in all tested forms – live cells, CFNS and heated CFNS, confirming the production of a thermostable compound(s), probably bacteriocin(s). Moreover, *Lbc. plantarum* DC 1246 caused antagonistic activity also towards *L. monocytogenes* strains (Lm-29 and Lm-30), but purely in the form of live cells. This specific antilisterial effect seemed to be caused by different compound(s), probably organic acids or other substances connected with live cells. In general, strains of *Lbc. plantarum* are considered to be stronger antilisterial agents in comparison with other lactobacilli (LOESSNER *et al.* 2003), although their application as biopreservatives must precede the study of potential production of biogenic amines (SHALABY 1996).

Bac⁺ *P. adicilactici* HV 12 showed antilisterial activity caused by different antimicrobial compounds, depending on each individual *L. monocytogenes* strain and experimental conditions (pH, heating). The

suppression of two *L. monocytogenes* strains (Lm-25 and Lm-26) by live cells and CNFS indicated organic acids and thermolabile compound(s) as antilisterial agents. Conversely, the inhibition of *L. monocytogenes* Lm-31 seemed to be caused mainly by an unspecified thermostable compound, probably pediocin-like substance (PLS) known as heat-stable (DRIDER *et al.* 2006). Obtained results coincided with the studies confirming *P. acidilactici* producing PLS as very effective antilisterial agents such as the whole class IIa bacteriocins (EIJSENK *et al.* 1998) causing permeabilisation of listeria cell membranes (HERRANZ *et al.* 2001). Different susceptibilities and resistance of some listerias to class IIa bacteriocins among *L. monocytogenes* strains could explain the highly strain-dependent antilisterial effect of *P. acidilactici* HV 12 determined in this study (GRAVESEN *et al.* 2002).

Ent. mundtii CCM 1282, previously confirmed as a producer of thermostable enterocin-like substance (ELS) (SOLICHOVÁ *et al.* 2012), showed the greatest antilisterial effect at all, namely against the five tested *L. monocytogenes* isolates (Lm-25, Lm-26, Lm-29, Lm-30, and Lm-31). In the present study, the thermostability of ELS was confirmed by significant antilisterial activity caused by heated CFNS of *Ent. mundtii* CCM 1282. The antilisterial effect of ELS-producing enterococci is explained by a close relationship between *Enterococcus* sp. and *Listeria* sp. (MORENO *et al.* 2006). On the other hand, while most enterococci are commensals, some of them are opportunistic human pathogens. From this point of view, enterococci are not generally considered as GRAS (MOELLERING 1992).

Moreover, the cheese starter culture DELVO-ADD® 100-X DSF (composed of an undefined mixture of lactococci and leuconostoc) previously confirmed as Nis⁻ and non-active in the inhibition of listerias (ŠVIRÁKOVÁ *et al.* 2009) showed significant antagonistic activity against five *L. monocytogenes* isolates (Lm-25, Lm-26, Lm-29, Lm-30 and Lm-31), namely in all tested forms – live cells, CFNS and heated CFNS. Surprisingly, it confirmed the production of a thermostable antilisterially active compound, although most starter cultures are not bacteriocin(s) producers (RATTANACHAIKUNSOPON & PHUMKHACHORN 2010). Obtained results could be applied in cheese technology, where the commonly used starter culture DELVO-ADD® 100-X DSF could play a natural protective role together with the main role in the technological process and beneficial influence on organoleptic and shelf-life characteristics.

CONCLUSIONS

Different sensitivity to the antilisterial potential of both individual LAB strains and cheese starter cultures among *L. monocytogenes* strains, originating from different sources, was shown. Either live cells or cell-free neutralized supernatant (CFNS) and/or heated CFNS of six individual LAB strains and one starter culture were highly effective in the suppression of at least one listeria strain. Isolates coming from raw milk and dairy equipment were significantly more sensitive to LAB in comparison with the reference collection strain (CCM 5576), which could represent a useful assumption in order to prevent the contamination by *L. monocytogenes* in dairy fermented foodstuffs and dairy industry. In general, the studied individual LAB strains and cheese starter cultures could be considered as a promising potential tool for biopreservation. For their practical application, some other studies will have to be performed in order to determine e.g. distribution of bacteriocin resistance, transmission of antibiotic resistance or production of biogenic amines among *L. monocytogenes* strains. Another necessity is the successful application of such biopreservative effect in chosen real foodstuff systems, which is more complicated and could influence the antilisterial activity of LAB because of the chemical composition and physical conditions of food environment.

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