Effect of *Lactococcus* sp. on the Growth of *Listeria* sp. in the Model UHT Milk System

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Abstract: The work was aimed at the growth suppression of cultured listerias strains by cultured lactococci strains or commercial mesophilic cheese cultures during common cultivations in the model UHT milk system (0.5% w/w of milk fat content) at 30°C during 18 h aerobically. Milk was primarily fermented by lactococci at the level of 10^8 CFU/ml and secondarily contaminated by listerias at the level of 10^3 CFU/ml. The most intensive growth suppressions of both *Listeria innocua* (CCM 5884 or Ln-03) strains were caused by *Lactococcus lactis* subsp. *lactis* (LCC 416 or CHCC 2281) strains or DELVO-ADD® 100-X DSF cheese culture; the listerias growth reductions was from the level of 10^3 CFU/ml to 10^0 CFU/ml. Obtained results should be applied to dairy industry provided that HACCP, GHP and GMP systems must be observed.

Keywords: *Lactococcus*; antilisterial activity; lactic acid; nisin; *Listeria*; UHT milk

Bacteria of *Listeria* genus attract an attention because of the health safety of final products, especially secondarily ripening cheeses, in the dairy industry of the Czech Republic and the European Union (EU). The majority of listerias species are harmless. *L. monocytogenes* (LM) species is pathogenic for human with 20% of death on listeriosis (Macela *et al*. 2006). *L. ivanovii* species is pathogenic just for sheep (Batt 2000). LM represents a facultative intracellular pathogen, which infects hosts by alimentary way (Schech 1983). Raw milk produced in milk farms represents a danger source of LM contamination as long as it is obtained during milking under GMP non-performance. In dairies raw milk is treated by heat with a guarantee LM inactivation. Final products can be secondarily contaminated by LM under conditions of sanitation regimes defaults or production control errors with the assistance of HACCP system. Listerias are able to reproduce at low storage temperatures and because of long incubation period of listeriosis it is difficult to prove whether a final product was contaminated at a producer or at a consumer only. For that reason naturally ways of listerias elimination are found. For example lactic acid bacteria can reduce or inhibit listerias and show rich biochemical activities.

There are various products with antilisterial effect on the market of the Czech Republic and the EU. Listex™ P100 is a well-known and described commercial product that contains a virulent strictly lytic phage of P100 type, which shows a high specificity against LM (Carlton 2005).

The aim of this work was to check a biochemical potential of lactococci for the reduction of listerias' growth in the model UHT milk system.

MATERIALS AND METHODS

Strains. Four cultured lactococcal strains of *Lc. lactis* subsp. *lactis* (LCC 416, NIZO R5, 303 and CHCC 2281), three commercial mesophilic mix cheese cultures (DELVO-TEC® LL 50A-Z

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DSF, DELVO-ADD® 100-X DSF and DELVO UX-11B DSF; O.K. Servis BioPro Ltd., Prague, Czech Republic) and four cultured listerias strains of *L. innocua* (CCM 5884, Ln-03 and Ln-10) and *L. monocytogenes* CCM 5576, which were stored in the Culture Collection of Bacteria, Yeasts and Moulds (Department of Dairy and Fat Technology, Institute of Chemical Technology Prague, Prague, the Czech Republic), were used for this work.

Cultivation conditions of used strains. Lactococci and cheese cultures were cultivated in M17 broth with lactose (0.5% w/w) (LM17 broth) at 30°C for 18 h aerobically. Listerias were cultivated in BHI broth at 30°C for 18 h aerobically. All strains were also cultivated in UHT milk (0.5% w/w of milk fat content) with/ or without addition of yeast extract (YE) (0.5% w/w) at 30°C for 18 h aerobically. For work were used 1% (v/w) inocula.

Estimation of lactococci and listerias colony counts. Lactococci colony count was estimated by an overlay plate method (CSN EN ISO 7218 2008); cultivation was in the LM17 agar at 30°C for 48 h aerobically. Listerias colony count was estimated by the overlay plate method; cultivation was in the BHI agar at 30°C for 48 h aerobically.

Estimation of listerias colony count on PALCAM agar. Listerias colony count was estimated by the plate method by a spread technique; cultivation was on a selective PALCAM agar at 30°C for 24 h (48 h) aerobically (CSN EN ISO 11290-2, Amendment A1 2005).

Estimation of minimal inhibition concentration of nisin (MIC_{nisin}) for tested strains. Sensitivity of tested strains to nisin was proved by the MIC_{nisin} determination. There was used following nisin concentration line (Nisaplin®, Danisco, Brabrand, Denmark) from 0 to 10 000 IU nisin/ml (e.g. from 0 to 1250 mg nisin/l).

Estimation of antilisterial activity of lactococci by agar spot method. Determination of antilisterial activity of lactococci was done as published by Schillinger *et al.* (1993) and cell-free supernatants were modified as published by Franz *et al.* (1996).

Common cultivation of lactococci and listerias in the model UTH milk system. Common cultivations of *Lc. lactis* subsp. *lactis* (LCC 416 nisin-producing strain, Nis^+, or CHCC 2281 nisin-non-producing strain, Nis^-) strains or DELVO-ADD® 100-X DSF cheese culture with *L. innocua* (CCM 5884 or Ln-03) strains were done in the model UHT milk system (0.5% w/w of milk fat content) at 30°C for 18 h aerobically. Characterisation of the first model cultivation system: lactococci at the level of 10^8 CFU/ml and listerias at the level of 10^3 CFU/ml were inoculated (1% v/w inoculum) to milk at the same time. Characterisation of the second model cultivation system: milk was fermented by lactococci only at the level of 10^8 CFU/ml and subsequently, only after fermentation, contaminated by listerias at the level of 10^3 CFU/ml.

RESULTS AND DISCUSSION

Lactococcal strains and mesophilic cheese cultures grew well in LM17 broth, UHT milk and UHT milk with YE addition; their counts at the level from 10^8 CFU/ml to 10^9 CFU/ml were determined in all tested cultivation media. Listerias strain grew well in BHI broth, UHT milk and UHT milk with YE addition; their counts at the level from 10^8 CFU/ml to 10^9 CFU/ml were determined in all tested media. YE addition did not cause any significant effects on the final colony counts of lactococci and listerias in comparison to milk without YE addition. Milk inoculated by lactococci and cheese cultures was standardly soured and on the contrary, milk inoculated by listeria was not soured.

Among lactococcal strains the highest sensitivity to nisin was determined in Nis^- *Lc. lactis* subsp. *lactis* CHCC 2281 strain and its MIC_{nisin} was 2.5 mg nisin/l (100 IU nisin/ml). Among cheese cultures the highest sensitivity to nisin was determined in Nis^- DELVO-ADD® 100-X DSF culture and its MIC_{nisin} was 0.08 mg nisin/l (3 IU nisin/ml). Among listeria strains the highest sensitivity to nisin was determined in *L. innocua* Ln-03 strains and its MIC_{nisin} was 2.5 mg nisin/l (100 IU nisin/ml). From literature it is known that Nis^+ lactococci contain genes in their genomes responsible for their immunity to nisin (Mills *et al.* 2006).

In tested lactococcal strains and cheese cultures were found out their antilisterial activities furthermore. Fresh lactococci cells and cheese cultures cells (A experiment), their cell-free supernatants adjusted at pH level of 6.5 (B experiment) and their cell-free supernatants adjusted at pH level of 6.5 with subsequent inactivation at 90°C for 5 min (C experiment), were tested. Results of antilisterial activities of lactococci and cheese cultures are presented in Table 1 and represent an average of three realised experiments.

Table 1. Antilisterial activity of lactococci cells against listerias (A experiment), antilisterial activity of lactococci cell-free supernatants against listerias (B experiment), antilisterial activity of modified lactococci cell-free supernatants against listerias (C experiment) – bacteria cultivation in BHI agar at 30°C for 18 h aerobically

Strain/culture	Activity against listerias											
	lactococci cells (A experiment)				lactococci cell-free supernatants* (B experiment)				modified lactococci cell-free supernatants** (C experiment)			
	CCM 5884	Ln-03	Ln-10	CCM 5576	CCM 5884	Ln-03	Ln-10	CCM 5576	CCM 5884	Ln-03	Ln-10	CCM 5576
LCC 416	+	+	+	+	+	+	–	–	–	–	–	–
NIZO R5	+	+	+	+	+	+	–	–	–	–	–	–
303	+	–	+	–	–	–	–	–	–	–	–	–
CHCC 2281	+	–	+	–	–	–	–	–	–	–	–	–
LL 50A-Z DSF	–	–	–	–	–	–	–	–	–	–	–	–
100-X DSF	–	–	–	–	–	–	–	–	–	–	–	–
UX-11B DSF	–	–	–	–	–	–	–	–	–	–	–	–

*Lactococci and cheese cultures supernatants adjusted at the pH level of 6.5; ** lactococci and cheese cultures supernatants adjusted at the pH level of 6.5 with subsequent inactivation at 90°C for 5 minutes

From A experiment results it is evident, that positive antilisterial activities were found out in Nis^+ *Lc. lactis* subsp. *lactis* (LCC 416 and NIZO R5) and Nis^- *Lc. lactis* subsp. *lactis* (303 and CHCC 2281) strains as well. Cheese cultures did not demonstrate any inhibition against listerias. *L. innocua* (CCM 5884 and Ln-10) strains were the most sensitive strains. From B experiment results it is possible to see, that antilisterial activity was found out only in Nis^+ strains, which inhibited both *L. innocua* (CCM 5884 and Ln-03) strains. From C experiment results it is obvious, that none listerial strain was inhibited by cell-free supernatants adjusted at pH specific level with subsequent thermal inactivation. This could be explained by decrease of inhibition effect caused by produced organic acids amounts because of cell-free supernatants adjustment at pH level of 6.5. Adjustment of pH had a significant effect on nisin activity, because of pH value for active nisin impact ranges from 3 to 6. Cell-free supernatants adjusted at pH specific level demonstrated decreased nisin activity. From literature it is known, that nisin is irreversibly inactive at pH 6.5 and higher (Hurst 1981).

The growth suppressions of both *L. innocua* (CCM 5884 or Ln-03) strains were caused by *Lc. lactis* subsp. *lactis* LCC 416 strain (nisin-producing strain) during common cultivations in UHT milk, which was inoculated by lactococci and listerias at the same time; listerias growth reductions were from the level of 10^3 CFU/ml to 10^1 CFU/ml.

The growth suspensions of both *L. innocua* (CCM 5884 or Ln-03) strains were caused by *Lc. lactis* subsp. *lactis* CHCC 2281 strain or DELVO-ADD® 100-X DSF cheese culture (nisin-non-producing strain and culture) during common cultivations in UHT milk, which was inoculated by lactococci and listerias at the same time; listerias remained stable at the level of 10^3 CFU/ml.

The most intensive growth suppressions of both *L. innocua* (CCM 5884 or Ln-03) strains were caused by *Lc. lactis* subsp. *lactis* (LCC 416 or CHCC 2281) strains or DELVO-ADD® 100-X DSF cheese culture during cultivations in UHT milk, which had already fermented by lactococci and subsequently contaminated by listerias; significant listerias growth reductions were from the level of 10^3 CFU/ml to 10^0 CFU/ml.

CONCLUSIONS

It is possible to use lactococci for the production of fermented dairy products in the form of mesophilic starter cultures for effective control of listerias occurrence and growth due to their rich biochemical activities, especially production of

lactic acid and various bacteriocins, including nisin. Results from model UHT milk systems confirmed that listerias were able to adapt to acid conditions (pH from 4.0 to 4.5) and their subsequent densities were neither higher nor lower provided when milk was contaminated by listerias before previous milk fermentation by lactococci. A pH leap change reduced number of listerias provided that already fermented milk was contaminated by listerias. Obtained results should be applied to dairy industry provided that HACCP, GHP and GMP systems must be observed.

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