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Coculture-inducible bacteriocin biosynthesis of different probiotic strains by dairy starter culture *Lactococcus lactis*

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Summary

Bacteriocins produced by probiotic strains effectively contribute to colonization ability of probiotic strains and facilitate their establishment in the competitive gut environment and also protect the gut from gastrointestinal pathogens. Moreover, bacteriocins have received considerable attention due to their potential application as biopreservatives, especially in dairy industry. Hence, the objective of this research was to investigate antimicrobial activity of probiotic strains *Lactobacillus helveticus* M92, *Lactobacillus plantarum* L4 and *Enterococcus faecium* L3, with special focus on their bacteriocinogenic activity directed towards representatives of the same or related bacterial species, and towards distant microorganisms including potential food contaminants or causative agents of gut infections. In order to induce bacteriocin production, probiotic cells were cocultivated with *Lactococcus lactis* subsp. *lactis* LMG 9450, one of the most important starter cultures in cheese production. The presence of bacteriocin coding genes was investigated by PCR amplification with sequence-specific primers for helveticin and was confirmed for probiotic strain *L. helveticus* M92. All examined probiotic strains have shown bacteriocinogenic activity against *Staphylococcus aureus* 3048, *Staphylococcus aureus* K-144, *Escherichia coli* 3014, *Salmonella enterica* serovar Typhimurium FP1, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* TM2, which is an important functional treat of probiotic strains significant in competitive exclusion mechanism which provides selective advantage of probiotic strains against undesirable microorganisms in gastrointestinal tract of the host. According to obtained results, living cells of starter culture *Lc. lactis* subsp. *lactis* LMG 9450 induced bacteriocin production by examined probiotic strains but starter culture itself was not sensitive to bacteriocin activity.

Key words: antimicrobial activity, bacteriocins, induction of bacteriocin biosynthesis, probiotics

Introduction

In fermented foods, lactic acid bacteria (LAB) display numerous antimicrobial activities (De Vuyst and Leroy, 2007; Šušković et al., 2010). Several LAB, mostly *Lactobacillus* strains, are also found in the gastrointestinal tracts of humans and animals and are considered to exert health-promoting effects which include antimicrobial activity against pathogen strains. This is mainly due to the production of organic acids, hydrogen peroxide, ethanol, diacetyl, acetaldehyde, but also of other compounds, such as antifungal peptides and bacteriocins. Bacteriocins are ribosomally-synthesized peptides or proteins with antimicrobial activity directed towards closely related Gram-positive bacteria, whereas producer cells are immune to their own bacteriocins (Collins et al., 2009). Bacteriocins derived from LAB in particular have a wide variety of potential applications such as, for example food biopreservatives and perhaps more interesting, as alternatives to antibiotics for medical and veterinary use (Cotter et al.,

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2005; Šušković et al., 2010). Antimicrobial activity is one of the most important functional properties of probiotic strains, because bacteriocin biosynthesis by probiotics can contribute to competitive exclusion of undesirable bacteria during host colonization, thereby helping to prevent host from pathogen proliferation. So far, bacteriocins produced by *Lactobacillus* strains are most common between LAB (De Vuyst and Leroy, 2007). There are several factors that can influence bacteriocin production: pH, temperature, ethanol concentration in specific microenvironment and growth conditions (Cotter et al., 2005; Rojo-Bezares et al., 2007). However, the effect of the presence of competing microorganisms on bacteriocin production is not well known yet, but it seems to have the key influence. Hence, the aim of this research was firstly to investigate antimicrobial activity of the cell-free supernatants (CFS) prepared from probiotic strains in different phases of bacteriocin purification towards representatives of the same or related bacterial species, and towards distant microorganisms including potential food contaminants or causative agents of gut infections. Furthermore, the interest was to find out whether bacteriocin production by probiotic strains *L. helveticus* M92, *L. plantarum* L4 and *E. faecium* L3 could be enhanced by coculturing with starter culture *Lc. lactis* subsp. *lactis* LMG 9450.

Materials and methods

Bacterial strains and media

Three selected probiotic strains, *L. helveticus* M92, *L. plantarum* L4, and *E. faecium* L3 were isolated and characterized as probiotics in the Laboratory of Antibiotics, Enzymes, Probiotics and Starter Cultures Technology, Faculty of Food Technology and Biotechnology University of Zagreb. All LAB strains were stored at -70 °C in the DeManRogosa Sharpe (MRS) broth (Difco) with 15 % (v/v) glycerol. Before the experimental use, these cultures were sub-cultured twice in the MRS broth. Three selected probiotic strains were examined for their antagonistic activities against the following indicator strains: *Staphylococcus aureus* 3048, *Staphylococcus aureus* K-144, *Escherichia coli* 3014, *Salmonella enterica* serovar Typhimurium FP1, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* TM2, as well as against representatives of the same and related LAB strains: *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus rhamnosus* GG and *Leuconostoc mesenteroides* LMG 7954, *Enterococcus faecium* LMG 9430, *Enterococcus faecium* A7, *Lactobacillus plantarum* Z88 and *Lactobacillus fermentum* A8. All indicator strains were derived from culture collection of the Laboratory of Antibiotics, Enzymes, Probiotics and Starter Cultures Technology, Faculty of Food Technology and Biotechnology University of Zagreb.

Preparation of cell free supernatants for antimicrobial activity assays

The antagonistic activity of the cell-free supernatants (CFS) of probiotic strains *L. helveticus* M92, *L. plantarum* L4 and *E. faecium* L3 was tested by the agar well-diffusion method as described by Ammor et al. (2006). Overnight cultures of *L. helveticus* M92, *L. plantarum* L4 and *E. faecium* L3 were centrifuged at 3600 *g* for 10 minutes and supernatants were filter sterilized (0.22 μm pore size, Millipore Millex porosity filters). In order to eliminate the inhibitory effect of lactic acid towards indicator strains, pH values of CFSs of *L. helveticus* M92, *L. plantarum* L4 and *E. faecium* L3 were adjusted to pH 6.0 with 5 M NaOH and catalase (1 mg/mL) treated to exclude the inhibition effect of hydrogen peroxide. Thus prepared CFSs were additionally treated with Proteinase K to confirm the presence of proteinaceous antimicrobials named bacteriocins. Furthermore, CFSs were concentrated by ultrafiltration on Amicon stirred ultrafiltration cell with 5000 Da exclusion membrane pore size.

The plates inoculated with indicator strains and different samples of probiotic CFSs were incubated overnight at 37 °C. Indicator strains were chosen to represent a range of spoilage and pathogenic organisms that are of safety concern to the food industry. Moreover, antimicrobial activity was tested towards representatives of the same and related LAB species.

CFS of probiotic strain *L. helveticus* M92 was also treated with solid diammonium sulphate (DAS) to 65 % saturation. Mixture was stirred for 2 h at 4 °C and then left to precipitate overnight at 4 °C. After that, centrifugation at 8,000 g for 20 min at 4 °C followed. The precipitate was resuspended in 2 mL of 50 mM phosphate buffer (pH 6.0) and investigated for its antimicrobial activity.

After desalting of precipitate of CFSs by dialysis, present proteins were separated by gel- filtration through Sephadex G-25 column (50 cm x 0.9) with 6000-8000 MWCO and eluted by 50mM PBS (4 mL/h) during 24 h at 4 °C. Protein fractions were collected and the protein concentration was determined by Bradford assay. Just before antimicrobial activity assays, protein fractions were filter sterilized through 0.22 μm pore size filter.

Induction of bacteriocin activity

Bacteriocin activity of overnight cultures of *L. helveticus* M92, *L. plantarum* L4 and *E. faecium* L3 was induced with potential inducing bacterium *Lc. lactis* subsp. *lactis* LMG 9450 according to Rojo-Bezares et al. (2007). Probiotic strain and inducing bacterium were added to 50 mL of MRS broth at initial bacterial concentration of 10^8 CFU/mL of probiotic strain and 10^7 CFU/mL of *Lc. lactis* subsp. *lactis* LMG 9450. Overnight cultivation of probiotic bacteria without *Lc. lactis* subsp. *lactis* LMG 9450 as coculture served as control. Coculture of probiotic strains with autoclaved *Lc. Lactis* subsp. *lactis* LMG 9450 was performed as described above with slight modification that bacterial cells of inducing strain have been previously submitted to autoclave sterilization (121 °C, 20 min). Cell-free supernatant (CFS) of *Lc. lactis* subsp. *lactis* LMG 9450, without addition of probiotic strains, did not have antimicrobial activity against probiotic and indicator strains.

PCR amplification of bacteriocin genes

In an attempt to determine whether the selected strains carried genes for production of known bacteriocins, PCR analysis using primers specific for individual bacteriocin genes was made. Total DNA from probiotic strains was extracted as described by Hopwood et al. (1985). Identification of bacteriocin-encoding genes by PCR was performed as described by Remiger (1996).

Results and discussion

Probiotics are defined as viable microorganisms which, upon ingestion in sufficient amounts, exert health benefits to the host beyond inherent basic nutrition (Guarner and Schaafsma, 1998). Probiotics are most frequently characterized from lactic acid bacteria (LAB) species. Three LAB strains examined for bacteriocinogenic activity in this research were previously defined as probiotics: *L. helveticus* M92, *L. plantarum* L4 and *E. faecium* L3 (Brkić, 1995; Šušković, 1996; Kos, 2001; Kos et al., 2003; Frece et al., 2005; Kos et al., 2008). In order to be selected as a probiotic, certain microorganism needs to fulfil a number of criteria coming from three different aspects: general, technological and functional (Šušković et al., 2009; Beganović et al., 2011a; Beganović et al., 2011b). These three probiotic strains were previously successfully applied in production of fermented probiotic beverages from milk permeate enriched with whey retentate (Leboš Pavunc et al., 2009). Bacteriocinogenic activity as a part of antimicrobial activity of these strains is of special importance from the functional probiotic aspect, which are demanded to inhibit undesirable microorganisms, making antimicrobial activity one of the most important characteristic of probiotic cultures. Bacteriocins are of special interest, not only for their biopreservative potential in dairy industry where these bacteria are frequently applied, but also as alternative antimicrobial strategy instead of antibiotic application, due to the increased spreading of antibiotic resistance especially for the past two decades resulting from uncontrolled use of antibiotics (Cotter et al., 2005; Džidić et al., 2008).

Diversity of bacteriocins was investigated, but so far only nisin and pediocin have commercial application as pure substances. However, there is a great potential and interest for the application of bacteriocin-producing LAB in the prevention of food spoilage which contributes to the extension of the product's shelf life and also brings biopreservation of minimally-processed food, which is one of the major challenges for the food industry. The use of bacteriocin-producing LAB as protective strains or bacteriocins as purified or concentrated compounds as biopreservatives to control undesirable bacteria, remains primary focus of researches related to food safety and quality (Šušković et al., 2010). Hence, inside of probiotic research concept, antimicrobial activity of the probiotic strains *L. helveticus* M92, *L. plantarum* L4 and *E. faecium* L3 was investigated, with a special focus on bacteriocin production. An induction of bacteriocin biosynthesis was examined by cocultivation of probiotic strains with the most frequently used cheese starter culture *Lc. lactis* subsp. *lactis* LMG

9450. Antibacterial activity of probiotic strains, towards different indicator bacteria, representatives of pathogens and LAB of the same or related species, was assayed by agar-well diffusion test. Sensitivity of 14 bacterial strains from different genera to the antimicrobial compounds present in cell free supernatants (CFS) of the probiotic strains are presented in Tables 1 and 2.

Table 1. Antimicrobial activity of cell-free supernatants (CFS) neutralized to pH 6.0 and catalase treated, from probiotic strains *Lactobacillus helveticus* M92, *Lactobacillus plantarum* L4 and *Enterococcus faecium* L3, performed from monoculture (A) and performed from cocultivation with *Lc. lactis* subsp. *lactis* LMG 9450 (B), towards: a) pathogen strains and b) representatives of the same and related lactic acid bacterial species. Antibacterial activity tested by agar-well-diffusion assay

a)

Indicator strain	Cell-free probiotic culture supernatant (inhibition zones)					
	M92		L4		L3	
	A	B	A	B	A	B
Staphylococcus aureus 3048	-	+	-	+	-	+
Staphylococcus aureus K-144	-	+	-	+	-	+
Escherichia coli 3014	-	+	-	-	-	-
Salmonella Typhimurium FP1	+	+	-	-	-	-
Bacillus cereus TM2	-	+	-	+	-	-
Bacillus subtilis ATCC 6633	-	+	-	+	-	-

Legend: + presence of inhibition zone; - absence of inhibition zone; / test was not performed

b)

Indicator strain	Cell-free probiotic culture supernatant (inhibition zones)					
	M92		L4		L3	
	A	B	A	B	A	B
L. acidophilus ATCC 4356	-	-	-	-	-	-
L. helveticus M92	-	/	-	-	-	-
*L. plantarum*L4	-	+	-	/	-	+
E. faecium L3	-	+	-	+	-	/
L. rhamnosus GG	-	-	-	+	-	-
Lc. lactis subsp. *lactis* LMG 9450	-	-	-	-	-	-
E. faecium A7	-	+	-	+	-	+
L. fermentum A8	-	-	-	-	-	+

Legend: + presence of inhibition zone; - absence of inhibition zone; / test was not performed

Table 2. Antimicrobial activity of upper fraction containing compounds with MW> 5000 Da (B*) and lower fraction containing compounds with MW <5000 Da (B**) of neutralized CFSs from probiotic strains *Lactobacillus helveticus* M92, *Lactobacillus plantarum* L4 and *Enterococcus faecium* L3 after the induction of bacteriocin biosynthesis by cocultivation with *Lc. lactis* subsp. *lactis* LMG 9450, towards: a) pathogen strains and b) representatives of the same and related lactic acid bacterial species. Neutralized CFSs were concentrated on Amicon stirred ultra filtration cell with 5000 Da exclusion membrane pore size. Antibacterial activity was tested by agar-well-diffusion assay

a)

Indicator strain	Concentrated cell-free probiotic culture supernatant (inhibition zones/mm)					
	M92		L4		L3	
	B*	B**	B*	B**	B*	B**
Staphylococcus aureus 3048	18	-	12	-	15	10
Staphylococcus aureus K-144	17	-	10	-	16	12
Escherichia coli 3014	13	-	-	-	-	-
Salmonella Typhimurium FP1	21	-	-	-	-	-
Bacillus cereus TM2	15	-	11	-	-	-
Bacillus subtilis ATCC 6633	17	-	10	-	-	-

Legend: - absence of inhibition zone; / test was not performed

b)

Indicator strain	Concentrated cell-free probiotic culture supernatant (inhibition zones/mm)					
	M92		L4		L3	
	B*	B**	B*	B**	B*	B**
L. acidophilus ATCC 4356	-	-	-	-	-	-
L. helveticus M92	/	/	-	-	-	-
L. plantarum L4	9	-	/	/	9	-
E. faecium L3	9	-	14	10	/	/
E. faecium ATCC 9430	14	-	12	-	-	-
E. faecium A7	13	-	10	11	10	-
L. rhamnosus GG	-	-	10	-	12	-
Lc. lactis subsp. *lactis* LMG 9450	-	-	-	-	-	-
L. fermentum A8	-	-	-	-	11	-

Legend: - absence of inhibition zone; / test was not performed

The antimicrobial activity of aforesaid probiotic strains was demonstrated previously (Kos et al., 2008; Beganović et al., 2011a). This activity could be prescribed mainly to the lactic acid production. In fact, the decline in pH arising from the production of lactic acid can be enough to inhibit certain strains. Non-dissociated form of lactic acid triggers lower internal pH of the bacterial cell, which causes collapse in electrochemical proton gradient in sensitive bacteria, hence having a bacteriostatic or bacte-

ricidal effect (Kos, 2001; González et al., 2007). Other factors that could be involved in antibacterial activity of these probiotic strains are competition for substrates and production of other substances with bactericidal or bacteriostatic action, including bacteriocins. Different factors can influence bacteriocin production such as pH, temperature, growth condition, presence of other microorganisms (Rojo-Bezares et al., 2007). Close contact of the competing microorganism has been reported as the most important factor (Barefoot et al., 1994; Maldonado et al., 2004; Rojo-Bezares et al., 2007).

Hence, in this research, the effect of coculture of probiotic strains with *Lc. lactis* subsp. *lactis* LMG 9450 on bacteriocin production was investigated. Antimicrobial activity of cell-free supernatants (CFS) of probiotic strains were performed both after growth of each strain in monoculture and after the growth in coculture with *Lc. lactis* subsp. *lactis* LMG 9450. In order to eliminate inhibitory influence of the lactic acid or other organic acid produced by these probiotic strains, CFSs were neutralized to pH = 6.0. Neutralized CFSs derived from the monoculture of all three strains *L. helveticus* M92, *L. plantarum* L4 and *E. faecium* L3 did not demonstrate inhibitory activity towards indicator food contaminant bacteria and lactic acid bacteria with the exception of *Salmonella* Typhimurium FP1 inhibition by *L. helveticus* M92 (Table 1a). An enlargement of the inhibitory spectrum was observed when probiotic strains were cocultivated with *Lc. lactis* subsp. *lactis* LMG 9450, for spoilage or pathogenic bacteria as well as different lactic acid bacteria. CFS from probiotic strain *L. helveticus* M92 has shown enhanced inhibitory effect towards all indicator food contaminant bacteria, while CFSs from *L. plantarum* L4 and *E. faecium* L3 inhibited growth of Gram-positive, but not Gram-negative indicator strains. Although bacteriocin activity can be of either broad or narrow spectrum, they are usually active against closely related LAB and some other Gram-positive bacteria. However, there are examples of bacteriocins active against Gram-negative *Enterobacteriaceae*, important for the control of pathogens in food (Stern et al., 2006).

When CFSs of the examined probiotic strains were neutralized to pH=6.0 and catalase treated, inhibitory activity towards representatives of the same and related LAB species was not obtained (Table 1b). After cocultivation with *Lc. lactis* subsp. *lactis* LMG 9450, neutralized CFSs from each probiotic strain inhibited some of tested LAB species (Table 1b). In addition, antimicrobial activity was tested after coculturing with heat-killed inducing bacteria of *Lc. lactis* subsp. *lactis* LMG 9450, but dead bacteria did not show induction capacity for bacteriocin production of probiotics under these experimental conditions (data not shown). According to the results of this study, *Lc. lactis* subsp. *lactis* LMG 9450 cells should be alive to induce bacteriocin production by *L. helveticus* M92, *L. plantarum* L4 and *E. faecium* L3, since induction did not take place with autoclaved cells (121 °C, 20 min). These results were similar to those previously reported for other bacteriocin-producing strains (Maldonado et al., 2004; Rojo-Bezares et al., 2007). Maldonado et al. (2004) propose that the presence of specific bacteria acts as an environmental signal to switch bacteriocin production in *L. plantarum* NC8. A quorum-sensing mechanism mediated by PLNC8IF appears to be involved in this process. LAB produce bacteriocins in very low concentration, thereby following experiments were performed with neutralized CFSs from all three probiotic strains which have been ultrafiltrated through Amicon stirred ultrafiltration cell with 5000 Da molecular exclusion membrane pore size.

Antimicrobial activity of the both CFS fractions was tested towards different indicator bacterial strains (Table 2a and 2b). Results were interesting as concentrated CFS (upper fraction containing compounds MW >5000) of probiotic strain *L. helveticus* M92 showed antibacterial activity towards all spoilage indicator and pathogenic strains. All concentrated CFSs derived from the three probiotic strains showed antimicrobial activity against *S. aureus* 3048 and K-144, whereas only concentrated CFS from *L. helveticus* M92 inhibited Gram-negative *Enterobacteriaceae* strains, including *S.* Typhimurium FP1 (Table 2a). Fraction of supernatant containing compounds with MW higher than 5000 Da from *L. helveticus* M92 has also shown antimicrobial activity to the representatives of *E. faecium* strains and against *L. plantarum* L4 (Table 2b). According to the results presented in Table 2b, inhibition of some LAB indicator strains was also present in concentrated CFS from *L. plantarum* L4 and *E. faecium* L3 containing compounds with MW higher than 5000 Da which point at presence of bacteriocinogenic activity of

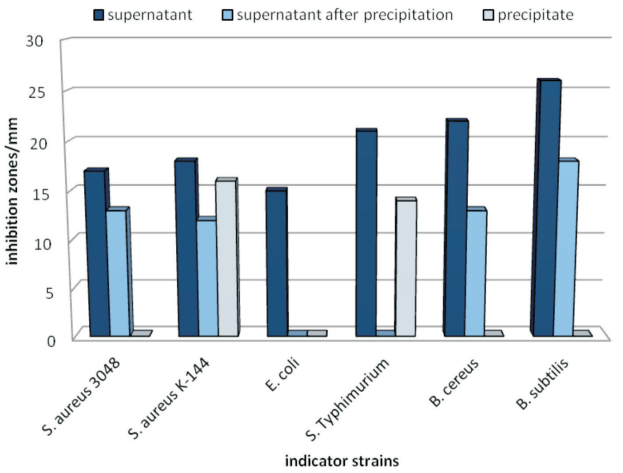

Figure 1. Antibacterial activity of cell-free supernatants of probiotic strain *Lactobacillus helveticus* M92, concentrated by precipitation with $(NH_4)_2SO_4$, tested by agar-well-diffusion assay

these strains. However, the broadest spectrum of antibacterial activity against indicator strains was shown by *L. helveticus* M92. Previously Kos et al. (2008) have demonstrated antibacterial activity of *L. helveticus* M92 against *Salmonella* through the competitive exclusion of *Salmonella* with probiotic strains *L. helveticus* M92 by *in vitro* and *in vivo* competition tests, since these results imply the role of bacteriocinogenic activity in this mechanism.

As this strain has shown the broadest antimicrobial spectra in comparison to the other two probiotic strain tested, including bacteriocinogenic activity against *S.* Typhimurium FP1, further concentration and purification of its bacteriocin was performed. Bacteriocins are protein substances so the next step was precipitation of proteins from CFS of *L. helveticus* M92 with diammonium sulphate (DAS). Afterwards antibacterial activity of the obtained protein precipitate was tested and compared to antimicrobial activity of supernatants before and after precipitation. The most interesting result was a strong inhibitory effect of concentrated proteins from CFS of *L. helveticus* M92 against *S.* Typhimurium FP1 (Figure 1). Furthermore, proteins from CFS of *L. helveticus* M92 were separated by gel-filtration on Sephadex G-25 column (Figure 2a). Only first five

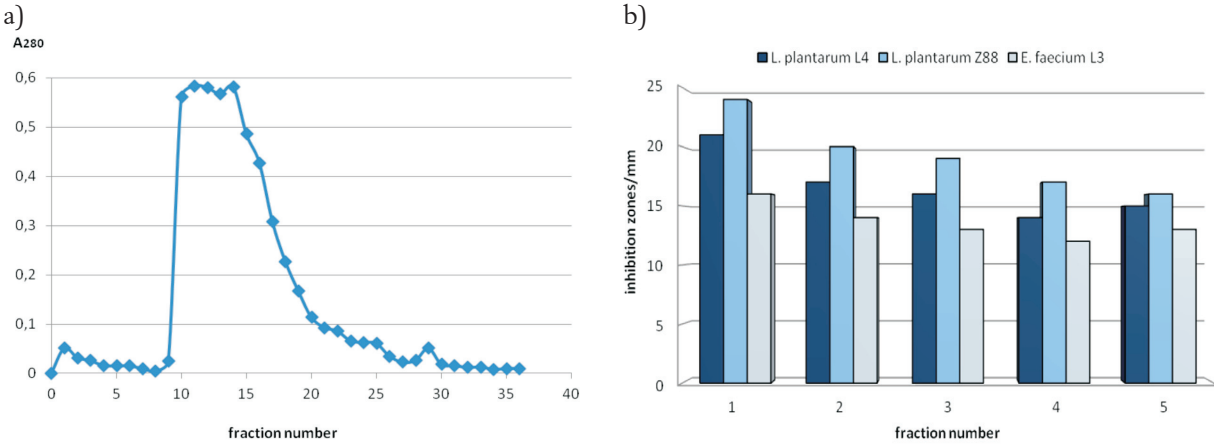

Figure 2. (a) Protein concentration in fractions obtained by gel-filtration chromatography after separation of proteins from *L. helveticus* M92 cell-free supernatant (CFS) concentrated with $(NH_4)_2SO_4$, on Sephadex G-25 colon and (b) antibacterial activity of first five active protein fractions. Antibacterial activity tested by agar-well-diffusion assay

Table 3. Stability of the active gel-filtration fraction from *L. helveticus* M92 cell-free supernatant (CFS) tested after heating, vacuum drying and 30 days of storage at 4 °C. Untreated active gel-filtration fraction was used as control. Antibacterial activity tested by agar-well-diffusion assay

Different treatments of *L. helveticus* M92	Inhibition zones (mm)		
CFS active gel-filtration fraction	*E. faecium* L3	*L. plantarum* L4	*L. plantarum* Z88
Control	16	21	23
Heat treatment at 100 °C/30 min	16	21	23
Vacuum drying (10 x concentrated sample)	20	26	28
Storage at +4 °C/30 days	16	21	23

protein fractions have shown antimicrobial activity against *L. plantarum* and *E. faecium* strains which were also sensitive to bacteriocin activity in previous experiments (Figure 2b, Tables 1 and 2). Sephadex G-25 column can separate proteins ranging in molecular weight from 100-5000 Da. Obtained results suggest that first five fractions contain the antibacterial substance with the molecular weight higher than 5000 Da (Figure 2a and 2b).

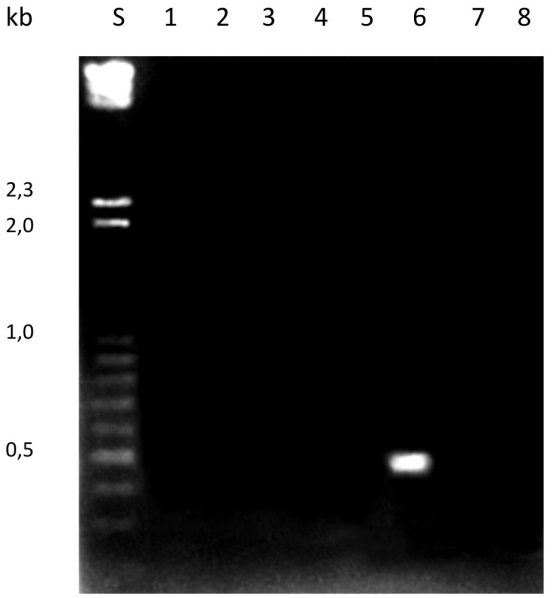

Figure 3. Gel electrophoresis of PCR fragment generated with helveticin specific primers from DNA of different lactic acid bacteria. Lanes: S - 100 bp DNA ladder + λ DNA/Hind III; 1- *L. acidophilus* ATCC 4365; 2- *E. faecium* A7; 3 - *L. plantarum* L4; 4 - *E. faecium* ATCC 9430; 5 - *E. faecium* L3; 6 - *L. helveticus* M92; 7 - *L. fermentum* A8; 8 - *Lc. lactis* subsp. *lactis* LMG 9450

Examination of the stability of active fractions has shown that bacteriocin of *L. helveticus* M92 was not sensitive to high temperature treatment, (100 °C/30 min) which is specific treat of small peptide bacteriocins of LAB, but was sensitive to Proteinase K treatment (Cotter et al., 2005). Active fraction retained the activity during 30 days storage at 4 °C and vacuum drying at 40 °C, which was shown as a suitable method for obtaining bacteriocin in concentrated form without losing its activity (Table 3).

Both examination of the inhibitory spectrum and characterization of the biochemical properties do not give sufficient information to distinguish different bacteriocins. Purification of bacteriocins to homogeneity is tedious and cumbersome. Therefore, Foulquie Moreno et al. (2003) suggested PCR as a first step to detect new bacteriocins. PCR analyses were performed to detect the presence of bacteriocin encoding genes in LAB strains. According to the obtained results, a PCR fragment was amplified from genomic DNA of *L. helveticus* M92. Nevertheless, negative results were obtained for *hlv* gene amplification with DNA isolated from other probiotic strains (Figure 3).

The presence of gene determinants for different bacteriocins by PCR has already been demonstrated for many lactic acid bacteria. According to Trmčić et al. (2011) autochthonous bacteriocinogenic *Enterococcus* strains were able to persist in mixed microbial cheese consortia. Besides biopreservative potential against undesirable bacterial species present in dairy products, bacteriocin production is often proposed as a beneficial characteristic of probiotic bacteria (Fooks and Gibson, 2002; Avonts et al., 2004). It may contribute to the colonisation resistance of the host and its protection against gastrointestinal pathogens (Reid et al., 2001).

Conclusions

hree different probiotic strains showed a diverse spectrum of antibacterial activity which includes inhibition of food-borne pathogens such as *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus*, *Bacillus subtilis* and *Bacillus cereus*. This activity is induced by the presence of living *Lc. lactis* subsp. *lactis* LMG 9450 cells. Especially promising are the results concerning antimicrobial effect of *L. helveticus* M92 against *S.* Thyphimurium FP1 which could be a tool for the control of this pathogen in food, especially in dairy products. Results of this research show potential usefulness of these probiotic strains and suggest further bacteriocin identification and application as food biopreservatives, especially in co-culture with *Lc. lactis* subsp. *lactis* which induce their bacteriocin biosynthesis. As confirmed in this study, presence of specific bacteria can act as an environmental signal to induce bacteriocin production in tested probiotic strains. The use of either antimicrobial substances as food additives or use of the bacteriocin-producing strains as starters or protective cultures, might contribute to the production of a safer and healthier traditional fermented dairy products. Also, the use of bacteriocin-producing strains with an enhanced capacity of colonisation and/or proliferation as starter cultures could improve the homogeneity and quality of the fermented food.

Poticanje biosinteze bakteriocina kod različitih probiotičkih sojeva kokulitvacijom s mljekarskom kulturom Lactococcus lactis

Sažetak

Biosinteza bakteriocina pomoću probiotičkih sojeva doprinosi njihovoj kolonizaciji epitelnih stanica domaćina, njegovoj zaštiti od patogena te uspostavljanju probiotičkog soja u kompetitivnom okolišu prisutnom u gastrointestinalnom sustavu domaćina, a osobito se istražuje njihova bakteriocinska aktivnost važna za primjenu kao biokonzervansa, posebno u proizvodnji mliječnih proizvoda. Stoga je cilj ovog istraživanja bio ispitati antimikrobno djelovanje probiotičkih sojeva *Lactobacillus helveticus* M92, *Lactobacillus plantarum* L4, *Enterococcus faecium* L3, s posebnim osvrtom na biosintezu bakteriocina. Antibakterijska aktivnost navedenih probiotičkih sojeva ispitana je prema predstavnicima istih i srodnih bakterijskih vrsta te prema različitim test mikroorganizmima, učestalim uzročnicima kvarenja hrane, te uzročnicima crijevnih infekcija. Istražena je i mogućnost induciranja biosinteze bakteriocina pomoću *Lactococcus lactis* subsp. *lactis* LMG 9450, jedne od najvažnijih starter kultura u proizvodnji sireva. Bakteriocinska aktivnost soja *Lactobacillus helveticus* M92 potvrđena je pomoću lančane reakcije polimerazom uz primjenu specifičnih početnica za gen koji kodira za bakteriocin helveticin. Svi probiotički sojevi su pokazali bakteriocinsko djelovanje prema *Staphylococcus aureus* 3048, *Staphylococcus aureus* K-144, *Escherichia coli* 3014, *Salmonella enterica* serovar Typhimurium FP1, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* TM2, što je važno funkcionalno svojstvo u mehanizmu kompetitivne ekskluzije koja probiotičkim sojevima omogućuje selektivnu prednost pred nepoželjnim mikroorganizmima u gastrointestinalnom sustavu domaćina. Prema dobivenim rezultatima žive stanice *Lc. lactis* subsp. *lactis* LMG 9450 induciraju bakteriocinsku aktivnost ispitivanih probiotičkih sojeva, a pri tome sama starter kultura nije bila osjetljiva na djelovanje bakteriocina.

Ključne riječi: antimikrobno djelovanje, bakteriocini, induciranje biosinteze bakteriocina, probiotici

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