Eur Food Res Technol (2007) 226:265–272
DOI 10.1007/s00217-006-0535-2

ORIGINAL PAPER

Screening of lactic acid bacteria for antimicrobial properties from mayonnaise-based products and raw materials

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Received: 17 March 2006 / Revised: 15 November 2006 / Accepted: 21 November 2006 / Published online: 10 January 2007

Abstract A collection of lactic acid bacteria isolated from both mayonnaise-based products and raw materials used to manufacture them was tested for antimicrobial activity. Out of 144 strains (97 lactobacilli, 23 lactococci and 24 enterococci) only three supernatants of *Enterococcus* spp. strains (EN3, EN14 and EN15) exhibited activity against lactobacilli and lactococci. The supernatant of the strain EN3 exclusively inhibited the growth of *Bacillus cereus* and *Listeria monocytogenes*. *Enterococcus* strains EN3, EN14 and EN15 produced thermostable bacteriocins, which had antibacterial activity.

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Keywords *Enterococcus* · Antibacterial · Bacteriocin · Mayonnaise

Abbreviations CFNS: Cell-free neutralised supernatant · LAB: Lactic acid bacteria

Introduction

Industrially made mayonnaises are safe products, due to the fact that only pasteurised egg yolks may be used to manufacture them. Mayonnaises also have a low acidity and a low water activity value. Home made mayonnaises contain nonpasteurised egg yolks and they usually have a higher pH value. Presence of pathogens cannot be excluded [1].

In industrially made mayonnaises, the main microbiological risk is the presence of yeasts (*Saccharomyces*, *Candida*, *Zygosaccharomyces*, *Debaryomyces*, *Pichia*), lactic acid bacteria and other bacteria (*Bacillus subtilis*, *B. pumilis*, *B. polymyxa* and *B. megaterium*), but the presence of pathogens (*Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella* spp.) cannot be excluded. Heterofermentative lactic acid bacteria, especially *Lactobacillus plantarum*, *Lbc. buchneri*, *Lbc. brevis*, *Lbc. casei* and *Lbc. fructivorans*, were isolated from delicacy products. These microorganisms cause sensory degradation of products (gas production, slime, texture changes, undesirable flavour, etc.) [2].

The sources of these microorganisms are raw materials, insufficient hygiene and poor sanitation [3]. What is more, lactic acid bacteria are generally tolerant to the addition of benzoic and sorbic acids (chemical food preservatives) [2].

In recent years, there has been a trend to use natural food products. Consumers no longer desire “unnatural” or

synthetic preservatives in their food but prefer a "safer" natural alternative. Therefore, new ways how to prevent the outgrowth of both spoilage and pathogenic organisms in food are being determined. As a result, there has been a focus on antimicrobial compounds produced by lactic acid bacteria (bacteriocins, organic acids, hydrogen peroxide, acetoin, carbon dioxide) [4, 5]. Bacteriocins are bacteria-produced antimicrobial proteinaceous compounds that have general bactericidal activity, often towards bacteria that are closely related to the bacteriocin-producing strain, as bacteriocin production is strain specific. Also, some bacteriocins inhibit the growth of pathogens and spoilage organisms during food processing and food fermentation [6]. Because LAB strains are 'generally recognized as safe' (GRAS) in food production use of either their bacteriocins or the bacteriocin-producing LAB starter cultures for food preservation has received much interest [7, 8]. Many different bacteriocins produced by enterococci have been described in details in literature, which is of great interest for food preservation against food-borne pathogenic bacteria and food spoilage bacteria [4, 9]. Enterococci are present in a variety of foods and in some of them, they also play a role in fermentation and ripening, and some strains offer a potential for use as starter, adjunct or protective cultures especially in the dairy industry, as well as in the meat industry [10]. Furthermore, some strains of *E. faecium* and *E. faecalis* are known probiotics and have been used with great success in commercial probiotic preparations [8].

Only one known research project is dedicated to studying the application of protective cultures in delicacy products (salad and dressing) [11], so the aim of this work was to isolate strains of lactic acid bacteria with antimicrobial activity from delicacy products and raw material used to manufacture them. These strains could, in the future, substitute chemical preservatives.

Materials and methods

Microorganisms and media

All microorganisms used, their growth conditions and sources are summarized in Table 1.

Table 1 Origin and incubation conditions of selected microorganisms

Strain	Medium	Conditions	Source
Lbc. sakei DMF 3017[a]	MRS	37 °C/18 h	University of Alberta, Edmonton, CA
Lbc. delbruckeii subsp. *lactis* LTI30	MRS	37 °C/18 h	DDFT, ICT Prague, CZ
Lbc. helveticus CH1	MRS	37 °C/18 h	Christian Hansen, DK
Lc. lactis subsp. *cremoris* MG 1614	M17	30 °C/18 h	AFRC, Great Britain
Lc. lactis subsp. *cremoris* NIZO B33	M17	30 °C/18 h	NIZO, The Netherlands
Lc. lactis subsp. *lactis* NIZO B643	M17	30 °C/18 h	NIZO, The Netherlands
Lc. lactis subsp. *lactis* NCDO 2054	M17	30 °C/18 h	NIZO, The Netherlands
Lc. lactis HMM 31	M17	30 °C/18 h	DDFT, ICT Prague, CZ
Enterococcus faecalis DMF 7051	Nutrient	37 °C/18 h	DBM, ICT Prague, CZ
Streptococcus uberis CCM4617	MRS	42 °C/18 h	Masaryk University in Brno, CZ
Geobacillus stearothermophilus DMF 2003	Nutrient	50 °C/18 h	Laktoflora, CZ
Bacillus cereus DMF 2001, *pumilis* DMF 2007	Nutrient	30 °C/18 h	DDFT, ICT Prague, CZ
Listeria monocytogenes CCM 5576	Nutrient	30 °C/18 h	Masaryk University in Brno, CZ
Staphylococcus aureus CCM 3953	Nutrient	30 °C/18 h	Masaryk University in Brno, CZ
Enterobacter cloacae DBM 3126	Nutrient	30 °C/18 h	DBM, ICT Prague, CZ
Pseudomonas fluorescens DBM 3056	Nutrient	30 °C/18 h	DBM, ICT Prague, CZ
Salmonella enterica subsp. *enterica* serovar *Typhimurium* CCM7205	Nutrient	37 °C/18 h	Masaryk University in Brno, CZ
Salmonella enterica subsp. *enterica* serovar *Enteritidis* CCM4420	Nutrient	37 °C/18 h	Masaryk University in Brno, CZ
Escherichia coli DMF 7502	Nutrient	30 °C/18 h	DBM, ICT Prague, CZ
Sacchammyces cerevisiae DMF1021	PDA	25 °C/2–5 days	DFCB, ICT Prague, CZ
Candida famata DMF 1001	PDA	25 °C/2–5 days	Laktoflora, CZ
Candida glabrata CBS 138	PDA	25 °C/2–5 days	CFRI, Budapest, H
Kluyveromyces marxianus var. *lactis* DMF 1004	PDA	25 °C/2–5 days	Laktoflora, CZ
Zygosaccharomyces rouxii CCM 8224	GMPY	25 °C/2–5 days	Masaryk University in Brno, CZ
Penicillium roqueforti DMF 0007	PDA	25 °C/2–5 days	Laktoflora, CZ
Penicillium expansum DMF 0005	PDA	25 °C/2–5 days	DDFT, ICT Prague, CZ
Aspergillus niger DMF 0801	PDA	25 °C/2–5 days	DDFT, ICT Prague, CZ
Geotrichum candidum DMF 0301	PDA	25 °C/2–5 days	DDFT, ICT Prague, CZ
Fusarium proliferatum DMF 4128	PDA	25 °C/2–5 days	DDFT, ICT Prague, CZ

[a] Anaerobically.

Lactic acid bacteria isolation

Samples were taken from mayonnaise, French dressing, Tartar sauce and raw materials used to manufacture them (water, parsley, egg yolk, mixture of vegetables, chilli spice, ketchup, green olives), diluted in a sterile saline solution and plated onto MRS and M17 agar (both Oxoid, Basingstoke, Hampshire, England). M17 agar was supplemented with lactose (Oxoid, Basingstoke, Hampshire, England), which was added separately after sterilization (950 ml of M17 agar and 50 ml of 0.28 mol l^{-1} lactose solution). The M17 plates were incubated at 30 °C for 48 h aerobically for isolation of lactococci and enterococci and the MRS plates were incubated 37 °C for 48 h anaerobically for isolation of lactobacilli. Colonies were selected at random from plates and the isolates purified by repeated streaking onto M17 or MRS agar [12].

Identification of isolates

Phenotypic genus-identification

The collection of 144 isolated strains was tested for catalase activity and Gram stained according to standard procedures [13]. Identification of the strains was conducted according to cell morphology; CO_2 production from glucose; and the growth at 10, 30, 37 and 45 °C; in the presence of 1.2 mol l^{-1} NaCl; and in MRS and M17 broth adjusted to pH 4.5 and 9.6, respectively, as described by Axelsson (1993) and Stiles and Holzapfel (1997) [14, 15]. Growth in the presence of 2.7 mmol l^{-1} methylene blue and on Slanetz–Bartley agar (Oxoid, Basingstoke, Hampshire, England) at 30 °C was also tested. Isolates with antimicrobial activity were identified using the ENCOCCUStest (Pliva–Lachema, a.s., The Czech Republic), by the production of pyrolidonyl arylamidase (PYRAtest, Pliva–Lachema, a.s., The Czech Republic), the motility and the production of pigment as well.

Genotypic genus-identification

Isolation of bacterial DNA For each individual *Enterococcus* spp. isolate, a 24-h culture from a blood agar, incubated aerobically at 37 °C, was used. The DNA isolation was carried out by boiling the bacterial suspension (density of 1 degree on the MacFarland scale) in sterile distilled water at 100 °C for 20 min with consecutive spinning at 18,130 × *g* for 10 min. Supernatant was used as a template [16].

Polymerase chain reaction Enterococcus spp. identification was carried out by a modification procedure described by Ke et al. [17]. The reaction was designed as a duplex-PCR, when, apart from the detection of the genus-specific *tuf*-gene (product size 112 bp), the internal control was also inserted. The sequence of the internal control was derived from the 16S rRNA gene (product size 241 bp) and is universal for any bacterial species [18]. The reaction mixture (25 μl) contained: 1 μl of the template, 12.5 μl PPT Master Mix (Top-Bio s.r.o., The Czech Republic) with 2.5 mM final concentration of $MgCl_2$, 1 μM primers Ent1 (5′-TACTGACAAACCATTCATGATG-3′) and Ent2 (5′-AACTTCGTCACCAACGCGAAC-3′), and 0.2 μM primers InKo1 (5′-GGAGGAAGGTGGGGATGACG-3′) and InKo2 (5′-ATGGTGTGACGGGCGGTGTG-3′) (Generi Biotech s.r.o., The Czech Republic). The strain *Enterococcus faecalis* CCM 4224 was used as the positive control, the strain *Streptococcus uberis* CCM 4617 was used as the negative control.

The PCR amplification was carried out in the thermocycler PTC-200 (MJ Research Watertown, USA) according to the following program: the initial denaturation 94 °C/2 min, amplification – 30 cycles (94 °C/30 s, 55 °C/15 s, 72 °C/30 s), final extension 72 °C/4 min. The products were analysed by gel electrophoresis in 20.0 g l^{-1} agarose (Serva Electrophoresis GmbH, Germany) with consecutive staining in ethidium bromide and visualization on a UV transilluminator. The DNA marker 155–970 bp (Top-Bio s.r.o., The Czech Republic) was used as the molecular weight standard.

Preparation of culture supernatant

Strains with antibacterial activity were grown in M17 broth for 18 h at 30 °C. The cultures were centrifuged at 3680 × *g* for 15 min, the cell-free supernatant was pH neutralized to pH 6.0–6.5 using NaOH (2.5 mol l^{-1} solution) and heated at 90 °C for 10 min to inactivate the remaining cells. The cell-free, neutralized supernatant (CFNS) was used in further bacteriocin characterization experiments [19].

Antibacterial activity assay

First, the antibacterial activity of the live cells was tested using 10 μl volumes of the 18-h culture from the M17 broth. The volumes (10 μl) were spotted onto the surface of an soft (7 g l^{-1}) M17, MRS or nutrient agar (7 ml, Oxoid, Basingstoke, Hampshire, England), which had been inoculated with 70 μl of an overnight culture of the indicator strain, which was diluted to the final concentration of 10^5–10^6 CFU ml^{-1}. The assay plates were incubated at 30, 37 or 50 °C for 24 h. The indicator microorganisms used for this assay are showed in the Table 1.

When some antibacterial activity was observed, the CFNS was tested using an agar spot test as described above [20].

Antifungal activity assay

The overlay method was performed using M17 plates on which 10 μl of LAB was inoculated in the middle of the

plate and incubated at 30 °C for 48 h. The plates were overlaid with 10 ml of soft PDA (Oxoid, Basingstoke, Hampshire, England) or soft GMPY agar containing 10^5 yeasts or moulds CFU ml^{-1}. The plates were then incubated aerobically at 25 °C for 2–5 days. The plates were examined for clear zones of inhibition around the bacterial streak [21].

Temperature stability of antibacterial compounds

To determine the temperature stability of antibacterial compound, 1 ml of CFNS was heated to 5, 15, 20, 30, 40, 50, 60, 70, 80, 90 and 100 °C in a water bath for 10, 30 and 60 min and 121 °C for 15 min in an autoclave. The residual activity was tested by agar spot test against the indicator strain *Lbc. sakei* DMF 3017 [12].

Effect of enzymes on the antibacterial activity

The CFNS (pH 7) was treated with various enzymes: catalase (2860 U mg^{-1}, Sigma–Aldrich, St. Louis, USA), lysozyme (58,100 U mg^{-1}, Sigma–Aldrich, St. Louis, USA), validase (fungal lipase, Valley Research, South Bend, USA) and proteolytic enzymes: proteinase K (52 U mg^{-1}, Sigma–Aldrich, St. Louis, USA), papain (50,000 NSU mg^{-1}, Gist-Brocades, Seclin Cedex, France), flavourzyme (a mixture of aminopeptidases, 500 MG, Novozymes A/S, Bagsveard, Denmark), protamex (a mixture of endoproteases and exoproteases, Novo Nordisk A/S, Bagsveard, Denmark), esperase (7.5 l FG, Novo Nordisk A/S, Bagsveard, Denmark) and α-chymotrypsin (51 U mg^{-1}, Sigma–Aldrich, St. Louis, USA). Each enzyme was dissolved in sterile demineralised water and added to the CFNS, to the final concentration of 1.0 mg ml^{-1}. The reaction mixture (1 ml) was incubated at 37 °C for 2 h and then heated to 100 °C for 10 min to inactivate the enzymes before assessing the remaining bacteriocin activity against the indicator strain *Lbc. sakei* DMF 3017 [22].

Results and discussion

Isolation and identification of isolates with antimicrobial activity

LAB strains (144) were isolated from delicacy products (mayonnaise, French dressing, Tartar sauce) and raw materials used to manufacture them (water, parsley, egg yolk, mixture of vegetables, chilli spice, ketchup, green olives). Catalase negative Gram-positive non-spore former strains were identified as referred above. The isolates were genus classified as follows: 97 lactobacilli, 23 lactococci and 24 enterococci (data not shown).

All isolated LAB were tested for antibacterial activity against several indicator strains: *Lbc. sakei* DMF 3017 [23, 24], *Lactobacillus helveticus* CH1 [25, 26] and *Lactobacillus delbrueckii* subsp. *lactis* LTI30 [27], which are sensitive to bacteriocins produced by LAB. First, the live cells were tested for antibacterial activity using agar spot test against the indicator strains and when some activity was observed, the CFNS of these strains were tested as well. *Lbc. sakei* was the most sensitive strain and it also caused food spoilage [28, 29], so this strain was used for other experiments. Only 48 strains out of 144 exhibited antibacterial activity against all used indicator strains, when their live cells were used (data not shown). These belonged to the genus *Lactobacillus* (38 strains), *Lactococcus* (2 strains) and *Enterococcus* (8 strains). When the CFNS was tested against the indicator strain *Lbc. sakei* DMF 3017 only three *Enterococcus* spp. strains were active. This can be explained by an acid effect or by a presence of thermolable compounds [30].

The strain EN3 was isolated from Tartar sauce, and strains EN14 and EN15 were isolated from chilli. Franz et al. [19] isolated LAB from a variety of vegetable products including raw vegetable, fermented vegetable and ready-to-eat salads (containing mayonnaise). They found a bacteriocin-producing strain *Enterococcus faecium* BFE 900 from black olives [19]. EN3, EN14 and EN15 were identified as *Enterococcus* spp. according to the results of the phenotypical and genotypical identification.

Genus identification was confirmed by the rapid PCR method based on the detection of *tuf*-gene occurred in *Enterococcus* species (*tuf*A-gen was proven to be presented in all tested *species*, which enables its successful application as the target sequence in the drafting of the genus-specific PCR method (Fig. 1) [17]. Detection of the genus-specific 112 bp product of *tuf*-gene was positive in *Enterococcus* spp. isolates and missing in the negative control *Streptococcus uberis* CCM 4617. Also Cupáková et al. [16] found this method specific and suitable for genus confirmation of enterococci. They successfully identified 283 *Enterococcus* spp. isolates from diverse origin [16].

Antibacterial activity of isolates

The biggest antibacterial activity against 20 heterofermentative lactobacilli isolated from Tartar sauce and mayonnaise was observed for the *Enterococcus* spp. EN3 strain. This strain was active against 19 heterofermentative lactobacilli strains. Strains *Enterococcus* spp. EN14 and EN15 were active against 14 heterofermentative lactobacilli (Table 2). The activity of enterococci against lactobacilli was previously described.

E. durans L28-2 inhibited *Lbc. sakei*, *Lbc. sanfranciscensis*, *Lbc. coryniformis* subsp. *coryniformis*, *Lbc. plantarum*, *Lbc. animalis*, *Lbc. pentosus*, *Lbc. gasseri*, *Lbc. rhamnosus*,

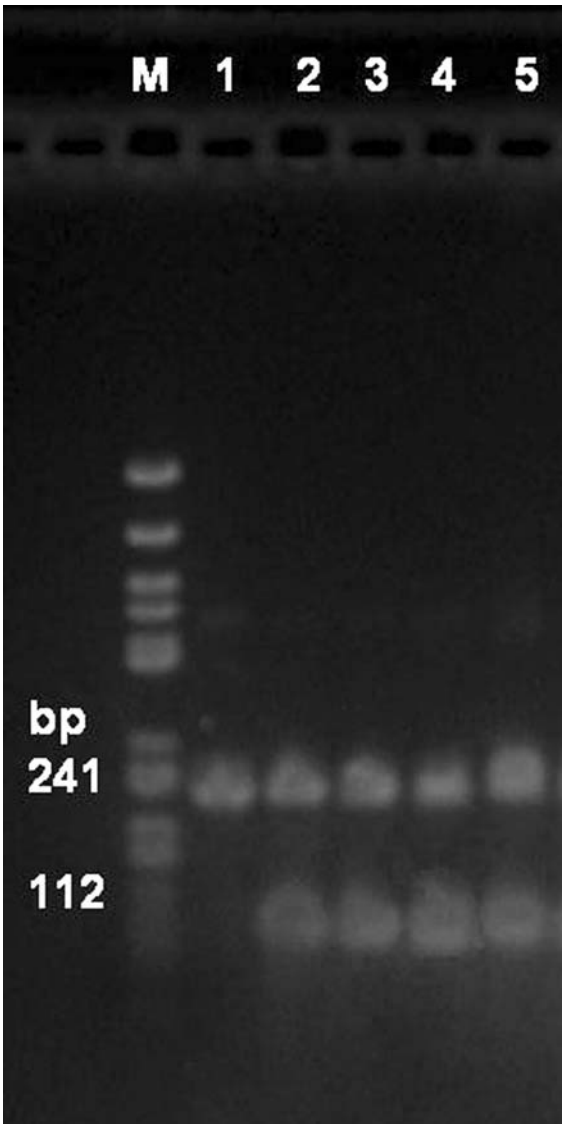

Fig. 1 Genus-specific identification of *Enterococcus* spp. strains by the duplex-PCR: line M – DNA marker 155–970; 1 – *Streptococcus uberis* CCM 4617, 2 – *Enterococcus faecalis* CCM 4224, 3 - *Enterococcus* spp. EN3; 4 – *Enterococcus* spp. EN14, 5 – *Enterococcus* spp. EN15

Lbc. helveticus and *Lbc. amylophilus* [31]. *E. faecium* 6T1a inhibited *Lbc. fermentum* and *Lbc. plantarum* [32], enterocin 012 produced by *E. gallinarum* inhibited growth of *Lbc. sakei* and *Lbc. acidophilus* [12], *E. faecium* P21 inhibited *Lbc. fermentum*, *Lbc. plantarum* and *Lbc. sakei* [33]. Enterocin P produced by *E. faecium* P13 inhibited *Lbc. curvatus*, *Lbc. sakei* and *Lbc. fermentum* [34]. *E. faecalis* BFE1071 only inhibited the strain *Lbc. salivarius* subsp. *salivarius* [35]. *E. faecium* JBL1083 [36], *E. faecium* BFE900 [19] and enterocin Q [37] only inhibited the growth of *Lbc. sakei*.

The antibacterial activity was tested against selected spoilage, food-borne and pathogen bacteria, which are usually present in mayonnaise-based products. Activity against other bacteria differed when the live cells and CFNS were

Table 2 Antimicrobial activity of *Enterococcus* spp. strains against heterofermentative lactobacilli

Indicator strains *Lactobacillus* spp.	Tested strains *Enterococcus* spp.		
	EN3	ENI4	EN15
H1	+/+	+/−	−/−
H2	+/−	−/−	−/−
H3	+/+	−/−	−/−
H4	+/+	+/−	−/−
H5	+/+	−/−	−/−
H6	+/+	−/−	−/−
M1	+/+	+/+	+/+
M3	+/+	+/+	+/+
2M2	+/+	+/+	+/+
2M3	+/+	+/+	+/+
2M4	+/+	+/+	+/+
2MB	+/+	+/+	+/+
T3	+/+	+/+	+/+
T4	+/+	+/+	+/+
T5	+/+	+/+	+/+
T6	+/+	+/+	+/+
T7	+/+	+/+	+/+
2T5	+/+	+/+	+/+
2T10	+/+	+/+	+/+
2T12	+/+	+/+	+/+

Note: Activity of live cells/activity of CFNS: −, no activity; +, activity (diameter of inhibition zone > 2 mm).

spotted on the agar inoculated with an indicator strain. In several cases when CFNS was applied, the activity was lost (Table 3). This was probably caused by the organic acids effect or by the thermolability of antibacterial compound [30]. *Enterococcus* spp. EN3 possessed the highest activity against other Gram-positive and Gram-negative bacteria. CFNS was active against *Lc. lactis* subsp. *cremoris* MG 1614, *Lc. lactis* subsp. *lactis* NIZO B643, *Lc. lactis* HMM 81, *Bacillus cereus* DMF 2001 and *Listeria monocytogenes* CCM 5576. *Bacillus cereus* and *Listeria monocytogenes* are food-borne bacteria, which are able to grow at low temperature and possess strong lipolytic and proteolytic activity. The CFNS from *Enterococcus* strains EN14 and EN15 were active only against the same lactococci as EN3. No inhibition between these three strains across each other was observed.

E. faecium 6T1a inhibited growth of *Lc. lactis* MG1614 [32], *E. faecium* EK13 [38] and *E. faecium* EF55 [39] inhibited growth of the *Lc. lactis* spp. strains. On the other hand, *E. faecium* JBL1061, JBL1083 and JBL1351 [36] did not inhibit growth of the *Lc. lactis* subsp. *lactis* strain. *Bacillus cereus* was inhibited by enterocin AS-48RJ [4] and *E. faecium* 6T1a [21], but was not inhibited by *E. faecalis* BFE1071 [35], *E. durans* L28-1 [31] and *E. faecium* JBL1061, JBL1083 and JBL1351 [36]. None of our strains was able to inhibit *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter cloacae* and *Salmonella enterica*. The same result has been observed for other enterococci [12, 33, 35, 36, 39–41]. *E. faecalis* was

Table 3 Inhibitory activity of *Enterococcus* spp. strains against lactic acid bacteria, food-borne pathogens and other indicator bacteria

Indicator strain	Tested strain *Enterococcus* spp.		
	EN3	EN14	EN15
Enterococcus spp.			
EN3	−/−	−/−	−/−
EN14	−/−	−/−	−/−
EN15	−/−	−/−	−/−
Lactococcus spp.			
Lc. lactis subsp. *cremoris* MG 1614	+/+	+/+	+/+
Lc. lactis subsp. *cremoris* NIZO B33	+/−	+/−	+/−
Lc. lactis subsp. *lactis* NIZO B643	+/+	+/+	+/+
Lc. lactis subsp. *lactis* NCDO 2054	−/−	−/−	−/−
Lc. lactis HMM 81	+/+	+/+	+/+
Other Gram-positive			
Enterococcus faecalis DMF 7051	−/−	−/−	−/−
Geobacillus stearothermophilus DMF 2003	+/−	−/−	−/−
Bacillus cereus DMF 2001	+/+	+/−	+/−
Bacillus pumilis DMF2007	+/+	+/−	+/+
Listeria monocytogenes CCM 5576	+/+	−/−	−/−
Staphylococcus aureus CCM 3953	−/−	−/−	−/−
Gram-negative			
Enterobacter cloacae DBM 3126	−/−	−/−	−/−
Pseudomonas fluorescens DBM 3056	+/−	−/−	−/−
Escherichia coli DMF 7502	−/−	−/−	−/−
Salmonella enterica subsp. *enterica* serovar *Typhimurium* CCM7205	−/−	−/−	−/−
Salmonella enterics subsp. *enterica* serovar *Enteritidis* CCM4420	−/−	−/−	−/−

Note: Activity of live cells/activity of CFNS: −, no activity; +, activity (diameter of inhibition zone > 2 mm).

not inhibited by enterocin AS-48RJ [4] and *E. casseliflavus* IM46K1 [40], but it was inhibited by *E. faecium* 6T1 [32]. *Listeria monocytogenes* was inhibited by enterocin P [34], enterocin AS-48RJ [4], enterocins A and B [42], *E. casseliflavus* IM46KI [40] and *E. faecium* EK13 [38]. *E. faecium* JBL1061, JBL1083 and JBL1351 inhibited eight strains of *Listeria monocytogenes*, but did not inhibit the growth of *Listeria monocytogenes* JBL1004 [36]. *Pseudomonas fluorescens* was not inhibited by *E. faecium* P21 [33].

Mayonnaise-based products are, also, often spoiled by yeasts and moulds [2], which is the reason why the antifungal spectrum of isolated enterococci with the antibacterial activity was also determined. *Enterococcus* spp. strains EN3, EN14 and EN15 had no antifungal activity against selected yeasts and moulds. This observation is in an agreement with data in literature [32, 43].

Effect of heating and enzyme treatment on the antibacterial compounds

Enterococcus strains EN3, EN14 and EN15 produced very thermostable compounds. There was no change in antibacterial activity of the CFNS after heating it up to 100 °C for 1 h and also after sterilization (121 °C for 15 min).

The antimicrobial compound of the *Enterococcus* spp. strain EN3 was completely degraded only after treatment with esperase. The treatment of this CFNS with other proteolytic enzymes caused a decrease in its antibacterial activity. The antimicrobial compound of the *Enterococcus* spp. strain EN14 was sensitive to proteinase K, papain, protamex and esperase. The antimicrobial compound of the *Enterococcus* spp. strain EN15 was sensitive to proteinase K, papain, flavourzyme, protamex, esperase and α-chymotrypsin (Table 4).

The results suggested that *Enterococcus* strains EN3, EN14 and EN15 produce thermostable bacteriocin(s). Accordingly, a different profile of the enzyme effect on CFNS

Table 4 Effect of various enzymes on antibacterial activity of *Enterococcus* spp. strains

Enzyme	Tested strains *Enterococcus* spp.		
	EN3	EN14	EN15
Catalase	14	13	12
Validase	14	13	8
Lysozyme	14	13	11
Proteinase K	6	0	0
Papain	7	0	0
Flavourzyme	11	10	0
Protamex	6	0	0
Esperase	0	0	0
α-Chymotrypsin	9	6	0
Control	16	15	13

Note: Diameter of inhibition zone is measured in mm.

Control: CFNS.

of strains EN14 and EN15 and also different antibacterial spectrum of these two strains can be suggested that they produce different thermostable compounds. Many thermostable bacteriocins produced by enterococci are well known [9, 22, 33, 34, 40]. The antagonistic activity of all the CFNS was not inhibited by catalase, which indicated that the inhibition observed was not due to hydrogen peroxide. Also CFNS was minimally inactivated by lysozyme and validase, which suggested that the peptide did not contain or require a lipid or carbohydrate moiety for activity, but more lipolytic and sacharolytic enzymes have to be tested to confirm this finding. The activity of the bacteriocin produced by *E. faecium* GM-1 isolated from an infant was not completely lost after treatment with proteinase K and papain, the GM-1 bacteriocin produced a smaller zone of inhibition after treatment with these enzymes [22]. The same result was observed for bacteriocin EN3. Protease inactivation characteristics of the antagonistic compound EN15 compared well to the *E. faecalis* BFE1071 bacteriocin [35] as this bacteriocin was also inactivated by α-chymotrypsin, papain and proteinase K.

Conclusion

New strains of LAB (three enterococci) with antimicrobial activity were isolated out of a collection of 144 LAB strains from mayonnaise-based products and raw materials used to manufacture them. The strains EN3, EN14 and EN15 produced very thermostable compounds with a proteinaceous base, these compounds had antibacterial activity. Heterofermentative lactobacilli and lactococci belong among the sensitive strains. The supernatant of the strain EN3 also inhibited the food-borne *Bacillus cereus* and *Listeria monocytogenes* strains. Therefore, the strain EN3 appears to be the most promising strain for further application in mayonnaise-based products to protect them against food spoilage and *Listeria monocytogenes* hazards. More precise identification at species level and also characterization of antimicrobial compounds (the influence of growth condition, pH stability and detection of bacteriocin structural gene) of these three enterococci will follow in the very near future.

Acknowledgements This work was supported by grant no. CEZ: MSM 6046137305 of the Ministry of Education Youth and Sports of The Czech Republic.

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