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International Journal of Food Microbiology 83 (2003) 171–184
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Leuconostoc carnosum 4010 has the potential for use as a protective culture for vacuum-packed meats: culture isolation, bacteriocin identification, and meat application experiments

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Received 21 January 2002; received in revised form 17 July 2002; accepted 18 July 2002

Abstract

A new culture, *Leuconostoc carnosum* 4010, for biopreservation of vacuum-packed meats is described. The culture originated from bacteriocin-producing lactic acid bacteria (LAB) naturally present in vacuum-packed meat products. Approximately, 72,000 colonies were isolated from 48 different vacuum-packed meat products and examined for antibacterial activity. Bacteriocin-producing colonies were isolated from 46% of the packages examined. *Leuc. carnosum* was the predominant bacteriocin-producing strain and *Leuc. carnosum* 4010 was selected for further experiments because it showed strong antilisterial activity without producing any undesirable flavour components in meat products. For identification of the bacteriocins produced, partial purification was carried out by ammonium sulphate precipitation, dialysis, and cation exchange chromatography. SDS-PAGE analysis revealed two bands with inhibitory activity corresponding to molecular sizes of 4.6 and 5.3 kDa. N-terminal amino acid sequencing showed that *Leuc. carnosum* 4010 produced two bacteriocins highly similar or identical to leucocin A and leucocin C. Application experiments showed that the addition of 10^7 cfu/g *Leuc. carnosum* 4010 to a vacuum-packaged meat sausage immediately reduced the number of viable *Listeria monocytogenes* cells to a level below the detection limit and no increase of *L. monocytogenes* was observed during storage at 5 °C for 21 days. The results presented demonstrate that *Leuc. carnosum* 4010 is suitable as a new protective culture for cold-stored, cooked, sliced, and vacuum-packed meat products.

Keywords: *Leuconostoc carnosum*; Protective culture; Biopreservation; Bacteriocins; *Listeria monocytogenes*; Vacuum-packed meats products

1. Introduction

The antimicrobial effects of lactic acid bacteria (LAB) have been used for thousand of years in order to extend the shelf life of foods. Traditionally, LAB

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doi:10.1016/S0168-1605(02)00364-1

have been present in spontaneous fermentation of different foods. However, the use of LAB as starter cultures has become widespread in the manufacture of fermented vegetables, dairy, and meat products. The advantages of employing starter cultures are numerous and have been pointed out by several authors (Lücke and Earnshaw, 1991; Ray, 1992; Holzapfel et al., 1995; Caplice and Fitzgerald, 1999). Among the beneficial properties of LAB, production of organic acids, hydrogen peroxide, and bacteriocins are important for microbiological safety and stability by inhibiting pathogenic bacteria such as *Listeria monocytogenes* (Holzapfel et al., 1995; Stiles, 1996; Lahti et al., 2001).

In nonfermented products like vacuum-packed meat products, LAB become the dominant microflora during storage (Shaw and Harding, 1984; Schillinger and Lücke, 1987). It has been reported that the dominating LAB often cause spoilage of the meat product (Yang and Ray, 1994; Björkroth et al., 1998; Samelis et al., 2000). However, it has also been reported that LAB keep the meat product sensory fresh throughout the storage period (Bredholt et al., 1999). These meat products are eminent sources for cultures, which can be used for biopreservation. Among these LAB, some are able to inhibit *L. monocytogenes* in the meat products suggesting their use as protective cultures (Hugas et al., 1998; Bredholt et al., 1999, 2001). Several workers have demonstrated that LAB isolated from vacuum-packed meat products are able to produce bacteriocins and other antimicrobial compounds, thus increasing the microbiological safety of the meat products (Ahn and Stiles, 1990; Bredholt et al., 1999; Schöbitz et al., 1999). For example, several *Leuconostoc* species of meat origin produce antilisterial bacteriocins generally characterised as heat-stable, class II bacteriocins (Hastings et al., 1991, 1994; van Laack et al., 1992; Felix et al., 1994; Papathanasopoulos et al., 1998).

The aim of this work was to investigate the frequency of bacteriocin-producing LAB in vacuum-packed meat products and to evaluate their potential as protective cultures in vacuum-packed meat products. This study describes the selection of a suitable candidate as well as the bacteriocins being produced. Finally, successful application of this strain as a protective culture in a vacuum-packed, sliced, cooked sausage was demonstrated.

2. Materials and methods

2.1. Isolation and identification of bacteriocin-producing isolates from meat products

Commercially available sliced and vacuum-packed meat products such as ham, salami, cooked loin, and smoked bacon were included in the screening for bacteria with antibacterial activity. Only meat products with acceptable sensory properties at the end of the declared shelf life were analysed. Tenfold dilution series of each product were spread on brain heart infusion (BHI) (Difco, Detroit, MI, USA), de Man, Rogosa and Sharp broth (MRS) (Merck, Darmstadt, Germany) and All Purpose with Tween (APT) (Merck) agar plates and incubated at 10 °C for 5–7 days and at 30 °C for 1–2 days. Agar plates with approximately 50 colonies were overlaid with soft agar seeded with *L. monocytogenes* DMRICC 0005, *Brochotrix thermosphacta* DMRICC 0006, *Escherichia coli* DMRICC 4224, *Salmonella typhimurium* DMRICC 3695, or *Leuconostoc mesenteroides* spp. *mesenteroides* DMRICC 0015, respectively. The cultures were previously grown in BHI for 18–24 h at 30 °C except for *Leuc. mesenteroides* spp. *mesenteroides* DMRICC 0015, which was grown in MRS for 18–24 h at 30 °C. All culture numbers used above refer to the culture collection of the Danish Meat Research Institute, Roskilde, Denmark. Colonies with inhibitory activity against at least one of the five indicator strains were isolated for additional characterisation. The production of bacteriocin was identified by the agar spot assay (Spelhaug and Harlander, 1989) and the peptide identity was established by adding 5-μl proteinase K (P2308, Sigma, St. Louis, MO, USA) and pronase E (P5147, Sigma), respectively next to the producer organism before overlaying with the inoculated soft agar. Fermentation of carbohydrates was analysed by the API 50 CHL system (BioMérieux, Marcy-l'Etoile, France). For selected strains including *Leuconostoc carnosum* 4010, SDS-PAGE analyses of cell extracts were carried out by BCCM/LMG Culture collection (Laboratorium voor Microbiologie, Universiteit Gent, Belgium) according to the procedure described by Pot et al. (1994). The normalised and digitised protein patterns were numerically analysed and clustered with reference profiles from an

established culture collection including recent industrial isolates in the LAB database.

2.2. Analysis of flavour compounds produced by the isolates

The bacteriocin-producing isolates grown in BHI were harvested by centrifugation (12,500 × *g* for 10 min at 5 °C) and resuspended in physiological saline to obtain an optical density (OD_{620}) in the range from 0.3 to 0.5. This suspension (10 ml) was inoculated on the surface of cooked sliced ham (100 g) containing 3% (w/w) NaCl and 150 ppm nitrite. The inoculated samples were vacuum-packed and stored at 7 °C for 10 and 20 days. For each sample, the number of viable cells was enumerated on BHI agar after incubation for 5 days at 20 °C. Volatile compounds were extracted and separated by headspace gas chromatography with flame ionisation detection (HSGC-FID) as described by Hinrichsen and Pedersen (1995). The compounds were tentatively identified according to Kovats' Indices. The results were examined by principal component analysis (PCA) in the Unscrambler Programme (CAMO, Trondheim, Norway version 5.5, 1994).

2.3. Characterisation of the inhibitory spectrum of Leuc. carnosum 4010

The inhibitory spectrum of *Leuc. carnosum* 4010 was determined using the method described by Schillinger and Lücke (1989). Shortly, the producer organism *Leuc. carnosum* 4010 was initially propagated for 48 h at 20 °C in BHI and then spotted (5 μl) onto BHI or MRS agar plates with a reduced glucose concentration of 0.2% w/v (Merck), and incubated for 48 h at 20 °C. Then plates were overlaid with 7-ml soft agar (BHI or MRS) containing 0.7% w/v Bacto agar (Difco), with 100 μl of the indicator organism previously added, and incubated for 48 h at 20 °C. Plates were examined for the presence of clear zones of inhibition.

2.4. Extraction of antilisterial activity from meat products

The presence of antilisterial activity in different commercial packages of sliced meat products such as cooked ham (seven products), cooked loin (four products), "rullepølse" (sausage made of rolled meat, two products), saveloys (two products), cured smoked non-heat-treated ham (three products) and poultry (four products) were examined after storage for 3–5 weeks at 5 °C. Meat products were mixed with 0.02 M HCl (2:1, w/v) using a stomacher for 1 min. The suspension was centrifuged at 16,000 × *g* for 5 min at 5 °C, and the antilisterial activity of the cell-free supernatant (20 μl) was measured in a radial well diffusion assay (Tagg and McGiven, 1971) using BHI agar (Difco) seeded with 10^5 cfu/ml *L. monocytogenes* 0005, previously grown at 30 °C in BHI for 18–24 h. The assay was incubated at 20 °C for 18–20 h. The concentration giving an inhibition zone of ∅12 mm, minus the diameter of the well, was arbitrarily set to 100 BU/ml.

2.5. Production and purification of bacteriocins from Leuc. carnosum 4010

Bacteriocin production was performed in a 2-l fermentor (CMF 100 Lab Fermentor, Alfa-Laval Chemap) connected to an automatic pH controller. *Leuc. carnosum* 4010, previously grown in MRS broth (Oxoid, Basingstoke, Hampshire, England) at 20 °C for 48 h, was added to an initial concentration of approximately 10^5–10^6 cfu/ml and grown in 1.5-l acetate-free MRS (Jack et al., 1996) containing 5% (w/v) glucose (Merck). The growth was carried out at 20 °C, pH 6.5 and with agitation at 50 rpm for 48 h. Preliminary experiments showed that the production of bacteriocin was optimal at these conditions (unpublished results). During fermentation pH was kept constant by automatically adding 1 M hydrochloric acid and 1 M sodium hydroxide. Samples were removed at specified time intervals and assayed for bacterial growth (cfu/ml) and bacteriocin activity (AU/ml). For purification the bacteriocin preparation was harvested after 48 h. Catalase, 0.2 g/l, (Sigma) was added to degrade hydrogen peroxide and cells were removed by centrifugation at 16,300 × *g* for 10 min. The supernatant was sterile-filtered using a 0.22-μm pore size GP Express membrane filter (Millipore, Bedford, MA, USA) and stored at −80 °C until analysis of bacteriocin activity or further purification. For purification, aliquots of 40-ml supernatant was added 40% (w/v) ammonium sulphate (Merck), and held for 30 min with stirring in a water bath at 20 °C.

Table 1
Number of colonies with antibacterial activity and bacteriocin activity, respectively, against five different indicator strains

Indicator strain	Number of colonies with antibacterial activity		Frequency of colonies with antibacterial activity	Number of colonies with bacteriocin activity		Frequency of colonies with bacteriocin activity
	10 °C	30 °C	(%)	10 °C	30 °C	(%)
Listeria monocytogenes 0005	23	57	0.6	17	11	0.19
Leuconostoc mesenteroides 0015	7	11	0.1	8	4	0.08
Brochotrix thermospacta 0006	5	13	0.1	1	2	0.02
Salmonella typhimurium 3695	2	19	0.1	0	0	0.00
Escherichia coli 4224	0	16	0.1	0	0	0.00

The number of colonies isolated at 10 °C and at 30 °C is indicated. The frequency indicates the percentage of colonies with antibacterial activity and bacteriocin activity, respectively, in relation to the total amount of colonies examined for each indicator strain (14,400).

The samples were centrifuged at 19,600 × *g* for 30 min at 4 °C and the pellet was resuspended in 2-ml 0.2 M sodium phosphate buffer (Merk), pH 6.0. Further purification included dialysis using a Spectra/Por dialysis membrane with a 1-kDa cut-off (Spectrum Medical Industries, Rancho Dominguez, CA, USA) against 10 mM sodium acetate buffer (Merck), pH 4.5. The dialysate was added urea (Merck) to a final concentration of 6 M before cation exchange, then subjected to a SP-Sepharose Fast Flow cation exchange (Amersham Pharmacia Biotech AB, Uppsala, Sweden), equilibrated with 10 mM sodium acetate buffer (pH 4.5), using a flow rate of 1 ml/min. The bacteriocins were eluted using the same buffer added 0.6 M NaCl.

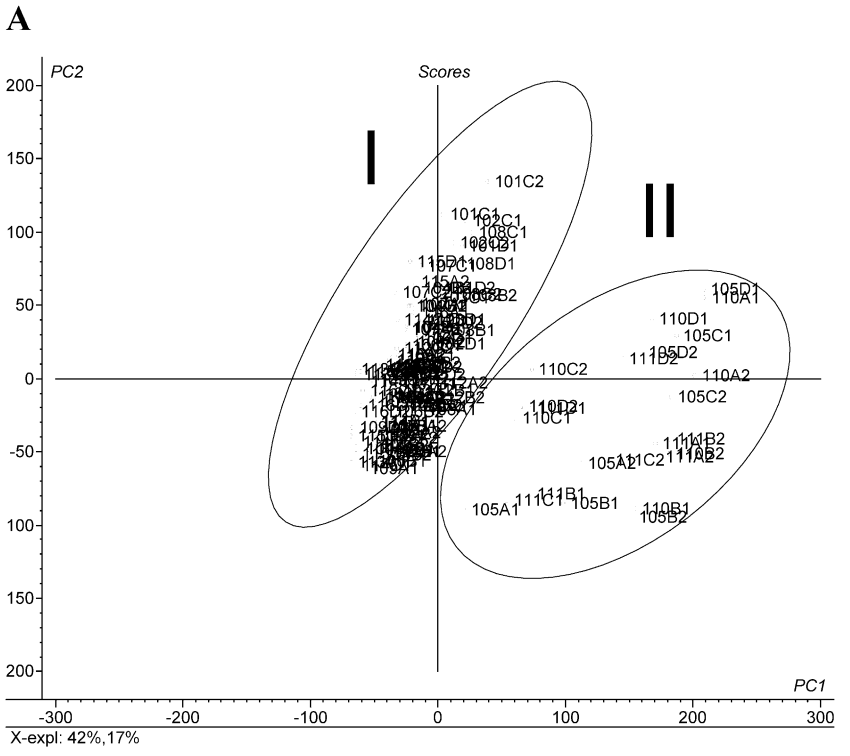
During purification, bacteriocin activity was measured in arbitrary units (AU/ml) using the microtiter assay described by Mörtved and Nes (1990) modified by using 150 μl instead of 20 μl of the indicator organism *Lactobacillus sakei* NCFB 2714 (OD_{620} = 0.1) and incubating at 37 °C for 4 h. Activity (AU/ml) was defined as the reciprocal of the highest twofold dilution showing 50% growth inhibition of the indicator organism measured as 50% of maximum turbidity in the microtiter plate assay system. Protein concentration of the bacteriocin fractions was determined by the BCA method (Pierce, Rockford, IL, USA) using bovine serum albumin A-3294 (Sigma) as standard.

2.6. Characterisation of bacteriocins from Leuc. carnosum 4010 by molecular mass determination and amino acid sequencing

The apparent molecular masses of leucocins 4010 were estimated by the Tricine-SDS-PAGE method (Schäger and von Jagow, 1987). Electrophoresis was performed at 100 V for 90 min using 10–20% gradient Tris–Tricine SDS-PAGE gels (Bio-Rad Laboratories, Hercules, CA, USA) and SeeBlue (Novex, San Diego, CA, USA) as molecular weight standard. Gels were either stained with Coomassie brilliant blue R-250 (Sigma) or stained and overlaid with the indicator organism *Lb. sakei* NCFB 2714 using the method described by Bhunia and Johnson (1992).

For amino acid sequencing, the eluate from cation exchange was applied to a reverse-phase (RP)-HPLC using a Nucleosil®300-5 C18 column, 25 × 0.46 cm (Macherey-Nagel, Düren, Germany) connected to an Alliance™ 2690 HPLC unit. Absorbance was measured at 280 nm with a 996 Photodiode Array Detector (Waters, Milford, MA, USA). Gradient elution was performed at 30 °C with a flow rate of 1 ml/min.

Fig. 1. Scores plot (A) and loading plot (B) after principal component analysis of volatile compounds produced by 14 LAB strains. In (A) the first three digits indicate the identity of the inocula (uninoculated control: 101, 102; *Leuc. mesenteroides* spp. *mesenteroides*: 103; *Leuc. carnosum*: 104, 107, 109, 113–116; *C. piscicola*: 105, 110, 111; *L. plantarum*: 112; unidentified LAB: 106, 108), the fourth digit indicates the incubation period (A, B: 10 days of incubation and C, D: 20 days of incubation) and the fifth digit designates replicate. The KI numbers in (B) refer to Kovats indices (KI 604: acetone; KI 588: ethanol; KI 736: 3-methylbutanal; KI 741: 2-methylbutanal; KI 629: 2-methylpropanal; KI 851: 3-methylbutanol; KI 853: 2-methylpropanol).


A
PC2
Scores
I
II
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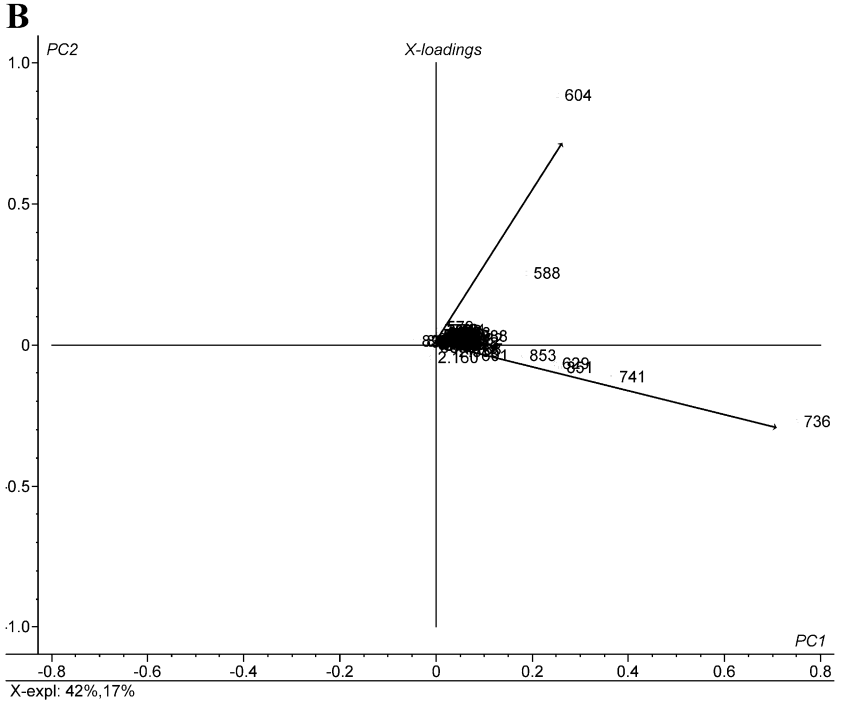
105B2
PC1
X-expl: 42%,17%


B
PC2
X-loadings
604
588
853
741
736
PC1
X-expl: 42%,17%

Elution buffer A contained 0.1% (v/v) trifluoroacetic acid (Merck) and buffer B contained 0.1% trifluoroacetic acid in acetonitrile (Merck). Elution was performed using a linear gradient from 100% of buffer A to 90% of buffer B in 50 min. Protein sequencing was carried out by automated amino terminal Edman degradation in a Procise 494 sequenator according to the recommendations of the manufacture (PE Biosystems, Foster City, CA). Cystine/half cystine residues were identified after alkylation on glass fibre according to Andrews and Dixon (1987).

2.7. Antilisterial activity of *Leuc. carnosum* 4010 in meat sausages

The ingredients of the pork meat sausages were shoulder, 51.8% (w/w); jowls, 18.0% (w/w); ice-water, 21.2% (w/w); potato starch, 3.7% (w/w); soya isolate, 1.4% (w/w); caseinate EM-HV, 1.4% (w/w); food-grade sodium polyphosphate N 15-15, 0.31% (w/w); sodium chloride, 2.0% (w/w); sodium nitrite, 60 ppm with all additives purchased at SFK (Avedøre, Denmark). The mixture was emulsified and sausages were pasteurised at 80 °C for 150 min with a centre temperature of 70 °C held for 50 min. Then sausages were cooled with cold water. The final sausages contained 15% fat (w/w) and 3% (w/w) sodium chloride in the aqueous phase. Sausage slices were inoculated with 3.0×10^4 CFU/cm^2 and 1.6×10^6 CFU/cm^2 (corresponding to 1.2×10^5 and 6.3×10^6 cfu/g, respectively) of *Leuc. carnosum* 4010, initially grown in BHI for 48 h at 20 °C, harvested by centrifugation and resuspended in peptone saline (1 g peptone/l, 9 g NaCl/l), and a cocktail of *L. monocytogenes* DMRICC 4124, *L. monocytogenes* DMRICC 4125, *L. monocytogenes* DMRICC 4126, *L. monocytogenes* DMRICC 4127 and *L. monocytogenes* DMRICC 4128, initially grown in BHI for 18–24 h at 30 °C and resuspended in peptone saline to obtain a total level of 10^4 CFU/g. The five strains of *L. monocytogenes* were mixed in equal amounts with regard to cell numbers. The sausage slices were vacuum-packed and stored at 5 °C for 28 days with sampling after 1, 7, 14, 21 and 28 days of incubation. Aerobic colony forming units were enumerated on BHI agar (Oxoid) after 5 days of incubation at 20 °C, and colony-forming units of *L. monocytogenes* were enumerated on Oxford agar (Oxoid) after 2 days of incubation at 37 °C. Triplicate determinations were performed.

3. Results

A total of approximately 72,000 colonies isolated from 48 different vacuum-packed meat products were examined for their antibacterial activity against one of the five indicator strains (Table 1). A total of 153 colonies (0.2%), showing antibacterial activity against at least one of the indicator organisms, were isolated. Antibacterial activity was detected more frequently at 30 °C compared to 10 °C with *L. monocytogenes* being the most frequently inhibited indicator strain (Table 1). Among the 153 colonies showing antibacterial activity, 97 bacteria were further characterized and 43 of them showed bacteriocin activity corresponding to 0.06% of the total number of colonies. The bacteriocin-producing colonies originated from 22 different packages out of 48 packages (46%) and were isolated from the agar plates with highest dilution of the meat samples indicating that the number of bacteriocin-producing bacteria may count approximately 10^6 cfu/g in the meat products. Among the 43 bacteriocin-producing bacteria, 27 strains of *Leuc. carnosum* were isolated from 16 packages, thus being the most frequently isolated bacteria. Another frequently isolated bacteria was *Carnobacterium piscicola* with six strains isolated from four different packages. Other bacteriocin-producing bacteria identified included six strains of *Leuc. mesenteroides* subsp. *mesenteroides*, two strains of *Lactobacillus plantarum* and two unidentified strains of LAB.

Fourteen of the isolated bacteriocin-producing lactic acid bacteria were further characterized for their ability to produce bacteriocin and flavour in cured cooked pork loin. Among these strains, eight strains produced extractable amounts of bacteriocin in cured cooked pork loin with the highest amount of bacteriocin (10–20 BU/g) obtained for the pork loin inoculated with four different *Leuc. carnosum* strains including *Leuc. carnosum* 4010 (results not shown). The inoculated samples contained between 7.8 and 8.5 log *Leuc. carnosum*/g whereas the control samples contained less than 2.0 log CFU/g after the incubation period (20 days at 7 °C). Principal component analysis of the peak areas of the volatile compounds

resulted in a model describing 42% of the variation by the first principal component and 17% by the second principal component (Fig. 1). The score plot shown in Fig. 1A reveals two groups designated as I and II. Group I from the scores plot includes the control samples as well as seven strains of *Leuc. carnosum*, one strain of *Leuc. mesenteroides* spp. *mesenteroides*, one strain of *L. plantarum* and two unidentified LAB

Table 2
Inhibitory activity of *Leuconostoc carnosum* 4010 against various indicator organisms

Indicator organisms	Source and strain	Growth conditions for inocula	Inhibition zone diameter (mm)
Lactobacillus acidophilus	LMG 13550	MRS, 48 h at 37 °C	0[a]
Lactobacillus bulgaricus	LMG 13551	MRS, 48 h at 42 °C	0[a]
Lactobacillus casei	LMG 13552	MRS, 24 h at 37 °C	0[a]
Lactobacillus curvatus	LMG 13553	MRS, 24 h at 30 °C	8[a]
Lactobacillus fermentum	LMG 13554	MRS, 24 h at 37 °C	0[a]
Lactobacillus helveticus	LMG 13555	MRS, 24 h at 42 °C	0[a]
Lactobacillus plantarum	LMG 13556	MRS, 24 h at 37 °C	0[a]
Lactobacillus reuteri	LMG 13557	MRS, 24 h at 37 °C	0[a]
Lactobacillus sakei	NCFB 2714	MRS, 24 h at 37 °C	26[a]
Lactobacillus sakei	LMG 13558	MRS, 48 h at 30 °C	26[a]
Pediococcus pentosaceus	LMG 13560	MRS, 24 h at 30 °C	17[a]
Pediococcus pentosaceus	LMG 13561	MRS, 48 h at 37 °C	0[a]
Leuconostoc cremoris	LMG 13562	MRS, 24 h at 25 °C	0[a]
Leuconostoc cremoris	LMG 13563	MRS, 48 h at 25 °C	10[a]
Leuconostoc mesenteroides spp. *mesenteroides*	DMRICC 0015	BHI, 48 h at 20 °C	28[b]
Streptococcus thermophilus	LMG 13564	MRS, 24 h at 42 °C	0[a]
Streptococcus thermophilus	LMG 13565	MRS, 24 h at 42 °C	0[a]
Propionibacterium acidipropionici	LMG 13572	GYP, 48 h at 30 °C	0[a]
Enterococcus faecalis	LMG 13566	BHI, 24 h at 37 °C	13[a]
Listeria innocua	LMG 13568	BHI, 24 h at 30 °C	20[a]
Listeria monocytogenes (serotype 4)	DMRICC 0005	BHI, 48 h at 20 °C	12[b]
Listeria monocytogenes (serotype 1)	DMRICC 4124	BHI, 48 h at 20 °C	20[b]
Listeria monocytogenes (serotype 1)	DMRICC 4125	BHI, 48 h at 20 °C	14[b]
Listeria monocytogenes (serotype 4)	DMRICC 4126	BHI, 48 h at 20 °C	18[b]
Listeria monocytogenes (serotype 4)	DMRICC 4127	BHI, 48 h at 20 °C	20[b]
Listeria monocytogenes (serotype 1)	DMRICC 4128	BHI, 48 h at 20 °C	14[b]
Listeria monocytogenes (serotype 1)	DMRICC 4140	BHI, 72 h at 20 °C	16[b]
Listeria monocytogenes	IVM 11137	BHI, 24 h at 30 °C	21[a]
Staphylococcus carnosum	LMG 13567	BHI, 24 h at 37 °C	0[a]
Staphylococcus aureus toxin A	IVM 2	BHI, 24 h at 30 °C	0[a]
Staphylococcus aureus toxin A + B	IVM 48	BHI, 24 h at 30 °C	0[a]
Brochotrix thermosphacta	DMRICC 0006	BHI, 48 h at 20 °C	0[b]
Bacillus cereus	LMG 13569	BHI, 24 h at 37 °C	0[a]
Clostridium sporogenes	LMG 13570	RCM, 24 h at 37 °C	0[a]
Salmonella infantis	IVM	BHI, 24 h at 30 °C	0[a]
Salmonella typhimurium	DMRICC 3695	BHI, 48 h at 20 °C	0[b]
Salmonella typhimurium	IVM	BHI, 24 h at 30 °C	0[a]
Escherichia coli	DMRICC 4224	BHI, 48 h at 20 °C	0[b]
Escherichia coli	IVM	BHI, 24 h at 30 °C	0[a]
Yersinia enterocolitica	IVM	BHI, 24 h at 30 °C	0[a]

Abbreviations: LMG: LMG Culture Collection, Laboratorium voor Microbiologie, Gent, Belgium; NCFB: National Collection of Food Bacteria, Aberdeen, Scotland; DMRICC: Danish Meat Research Institute Culture Collection; IVM: Institute of Veterinary Microbiology, The Royal Veterinary and Agricultural University, Copenhagen, Denmark. MRS: de Man, Rogosa and Sharp; BHI: Brain Heat Infusion; RCM: Reinforced Clostridia Media; GYP: glucose (5 g/l), yeast extract (3 g/l), peptone (10 g/l), meat extract (10 g/l), NaCl (5 g/l), pH 7.0.

[a] Agar spots were carried out on BHI agar.

[b] Agar spots were carried out on MRS agar.

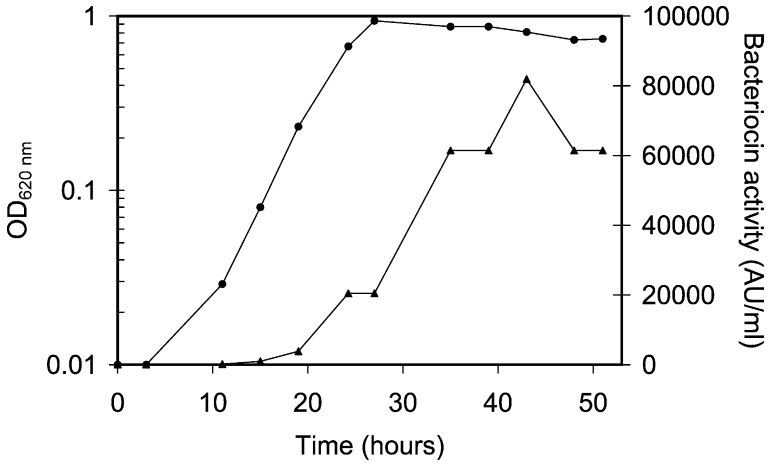


Fig. 2. Growth of *Leuc. carnosum* 4010 (●) and bacteriocin production (▲) at 20 °C and at a constant pH of 6.0 in acetate-free MRS added 5% (w/v) glucose.

strains. The loadings plot in Fig. 1B shows that the volatile compounds for this group consisted of acetone (KI 604) and ethanol (KI 588) with the highest amount found in the control samples. This indicates that strains belonging to group I do not produce any specific volatile compound compared to the uninoculated control samples. Group II contained three strains of *C. piscicola*, which produced volatile compounds such as 3-methylbutanal (KI 736), 2-methylbutanal (KI 741), 2-methylpropanal (KI 629), 3-methylbutanol (KI 851) and 2-methylpropanol (KI 853) (Fig. 1). Sensory evaluation of cooked sliced ham biopreserved with *Leuc. carnosum* 4010 or *C. piscicola* was carried out after storage for 1 and 4 weeks at 5 °C using triangular test. Samples inoculated with *Leuc. carnosum* were not significantly different from uninoculated controls whereas samples inoculated with *C. piscicola* differed from the uninoculated controls ($P < 0.01$) (results not shown).

Leuc. carnosum 4010, which was isolated from the surface of sliced vacuum-packed ham, was chosen for further experiments because it showed strong inhibition against *L. monocytogenes* and produced the highest amount of bacteriocin on the cooked sliced ham as mentioned above, without producing any undesirable flavour components according to the analysis of flavour compounds. As seen from Table 2, *Leuc. carnosum* 4010 was inhibitory against different strains of *L. monocytogenes* and against *Listeria innocua* and some LAB. No inhibitory activity was observed against *Staphylococcus aureus*, *B. thermosphacta*, *Bacillus cereus* and the Gram-negatives including *Salmonella infantis*, *S. typhimurium*, *E. coli* and *Yersinia enterocolitica* (Table 2).

The natural presence of compounds with antilisterial activity was examined in different commercial packages of sliced meat products after storage for 3–5 weeks at 5 °C. Extractable amounts of compounds

Table 3
Purification of leucocins 4010

Purification steps	Total activity (AU)	Total protein (mg)	Specific activity (AU/mg protein)	Activity recovery (%)	Purification fold
Culture supernatant	30,720,000	8805	3489	100	1
Ammonium sulphate precipitation	9,830,400	166	59,219	32	17
Dialysis	9,830,400	48	204,800	32	59
Ion-exchange, the eluate from SP-sepharose fast flow	4,915,200	4.4	1,117,091	16	320

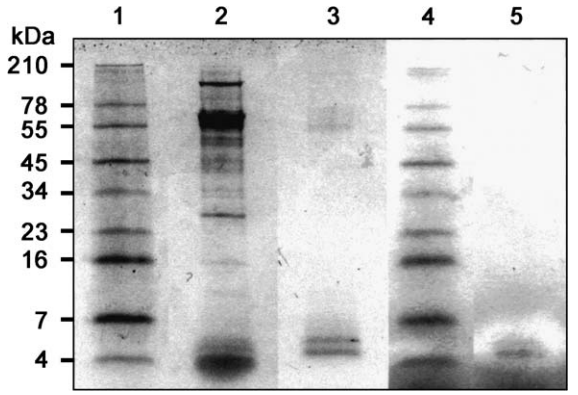


Fig. 3. SDS-PAGE of bacteriocins from *Leuconostoc carnosum* 4010 and detection of inhibitory activity. Coomassie brilliant blue stained gel: lane 1, standard; lane 2, ammonium sulphate precipitate; lane 3, purified leucocins from *Leuc. carnosum* 4010. Gel overlaid with MRS soft agar containing *Lb. sakei* NCFB 2714 and incubated for 18 h: lane 4, standard; lane 5, purified leucocins from *Leuc. carnosum* 4010.

with antilisterial activity were found in 18% of the packages including two packages of ham, one package of cooked loin and one package of rullepølse with concentrations between 7 and 55 BU/g (results not shown).

Growth of *Leuc. carnosum* 4010 in a 2-l fermentor resulted in an increase in cell density (OD-600 nm) from 0.01 to 0.94 during the first 27 h of growth and bacteriocin production was initiated during the exponential growth phase reaching the highest level (6–8×10^4 AU/ml) in the stationary growth phase (Fig. 2). After 48 h of growth, the supernatant was collected for purification. The efficiency of the various steps of purification is shown in Table 3. The purification steps resulted in more than a 300-fold increase in the specific activity and an activity recovery of 16%. Subsequently, SDS-PAGE analysis showed two bands corresponding to molecular sizes of 4.6 (lower band) and 5.3 (upper band) kDa and with inhibitory activity against *Lb. sakei* NCFB 2714 (Fig. 3). After blotting the SDS-PAGE gel to a PVDF membrane, the N-terminal 15 residues of the lower band showed peptides with two different amino acids in position 2, an asparagine and a tyrosine, respectively. For the upper band, N-terminal sequencing showed a peptide with only asparagine in position 2. Additional separation of the compounds using HPLC resulted in significant loss of bacteriocin activity. Therefore, SDS-PAGE analysis was used to reveal the presence of peptides with molecular sizes of 4.6–5.3 kDa in the HPLC-eluted fractions. Edman degradation of a peptide from a selected HPLC fraction was determined up to the 39 amino acid residues with asparagine in position 2. This peptide was designated leucocin B-4010 (Table 4). Another HPLC fraction was determined up to the 30 amino acid residues and it contained equal amounts of leucocin B-4010 and another peptide, designated leucocin A-4010. The sequence of leucocin A-4010 was obtained by subtracting sequence B from the mixture (Table 4).

Meat application experiments showed that *Leuc. carnosum* 4010 inhibited growth of *L. monocytogenes* in a cooked, sliced, and vacuum-packed meat sausage during storage at 5 °C for 28 days. The rate of inhibition depended on the initial concentration of *Leuc. carnosum* 4010 (Fig. 4). For sausages added

Table 4
Partial amino acid sequences of the bacteriocins leucocin A-4010 and B-4010 produced by *Leuconostoc carnosum* 4010 and amino acid sequence homology comparison to leucocin A-UAL 187 produced by *Leuconostoc gelidum* UAL 187 (Hastings et al., 1991), to leucocin B-TA11a produced by *Leuconostoc carnosum* Ta11a (Felix et al., 1994) and to leucocin C-TA33a produced by *Leuconostoc mesenteroides* TA33a (Papathanasopoulos et al., 1998)

Bacteriocin	Amino acid sequence	Number of amino acids
Leucocin A-4010	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLA----	>30
Leucocin A-UAL 187 or Leucocin B-TA11a	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW	37
Leucocin B-4010	KNYGNGVHCTKKGCSVDWGYAWTNIANNSVMNGLTGGNA----	>39
Leucocin C-TA33a	KNYGNGVHCTKKGCSVDWGYAATNIANNSVMNGLTG----	>36

Differences between amino acids are underlined. Unidentified amino acids are indicated by X.

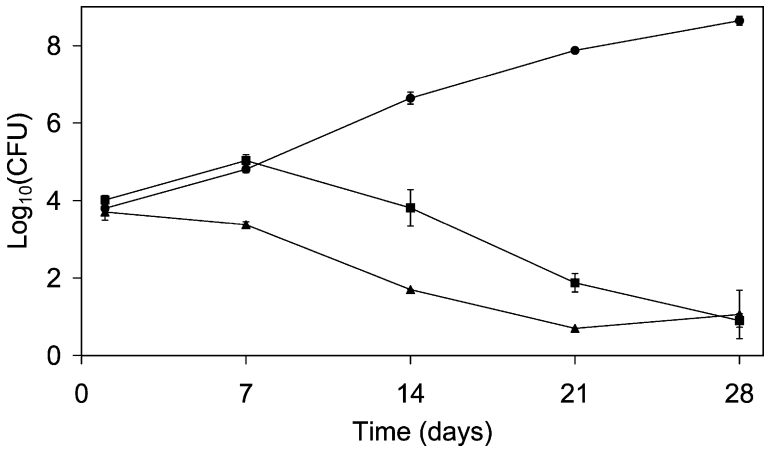


Fig. 4. The influence of *Leuc. carnosum* 4010 on the growth of a mixture of five different *L. monocytogenes* strains (total inoculation level 10^4 CFU/g) in a cooked, vacuum-packed and sliced meat sausage during storage at 5 °C (● no addition of *Leuc. carnosum* 4010, ■ initial addition of 1.2×10^5 cfu/g *Leuc. carnosum* 4010, ▲ initial addition of 6.3×10^6 cfu/g *Leuc. carnosum* 4010). Triplicate determinations were performed.

Leuc. carnosum 4010 at a level of 1.2×10^5 cfu/g, no inhibitory effect against *L. monocytogenes* was seen during the first week of storage. Continuous storage inhibited growth and led to a decrease of *L. monocytogenes* compared to sausage slices without *Leuc. carnosum* 4010 added. At the end of storage (28 days) the cell count of *L. monocytogenes* was reduced to a level below 10 cfu/g for sausages added *Leuc. carnosum* 4010 compared to a cell count of 10^8 cfu/g for sausage slices without *Leuc. carnosum* 4010 added. A higher initial concentration of *Leuc. carnosum* 4010 (6.3×10^6 cfu/g) caused an immediate reduction in the viable count of *L. monocytogenes*, resulting in a level below 10 cfu/g after 21 days of storage (Fig. 4). At the end of the storage period (28 days), two of the three packages contained a viable cell count of *L. monocytogenes*, which was below 10 cfu/g whereas the third package contained 60 cfu/g.

4. Discussion

This study showed that bacteriocin-producing LAB are widely distributed in commercially meat products. Among the analysed meat products, 46% of the packages contained bacteriocin-producing strains in high numbers (approximately 10^6 cfu/g). The frequency of bacteriocin producing strains isolated from foods differs in various studies. Coventry et al. (1997) examined 72 meat and dairy products at the time where the products were spoiled and found bacteriocin-producing strains in 46% of the samples, which is in accordance with the present study except that the products analysed in the present study were not spoiled at the end of the declared shelf life. However, Garver and Muriana (1993) only found bacteriocin-producing strains in 13% of the meat products analysed at the end of the declared shelf life. Among the LAB colonies isolated from meat products in the current study, approximately 0.2% showed antimicrobial activity whereas only 0.06% produced bacteriocin. Higher frequencies (0.2% and 0.9%) of bacteriocin producers among the total amount of LAB strains in meat and dairy products were reported by Coventry et al. (1997) and Garver and Muriana (1993), respectively. Differences in the frequencies of bacteriocin-producing strains could be related to the different meat products analysed, time for sampling as well as the identity and number of indicator strains used for analyses.

The percentage of meat products containing bacteriocin-producing strains (46% of the packages) was found to be higher than meat products containing extractable amounts of antilisterial activity (18% of the packages). One explanation might be that not all bacteriocin-producing strains are able to produce significant amounts of antilisterial compounds in the meat products, e.g. commercial meat products often

contain approximately 3% sodium chloride (aqueous phase) and sodium chloride is known to inhibit the production of some bacteriocins (Larsen et al., 1993). The presence of interfering substances extracted from meat, adsorption of bacteriocin on meat components, and degradation of bacteriocins in the meat matrix could be factors affecting the percentage of meat products containing extractable amounts of bacteriocin (Murray and Richard, 1997). In summary, the present study shows that bacteriocin-producing bacteria as well as extractable amounts of antilisterial compounds are frequently found in commercial meat products.

In the present study, *Leuc. carnosum* was identified as the predominant bacteriocin-producing bacteria from the vacuum-packed meat products. *Leuconostoc* and other heterofermentative LAB have commonly been found to predominate in vacuum-packed, non-smoked and cooked ham or luncheon meat (Shaw and Harding, 1984, 1989; Yang and Ray, 1994; Björkroth et al., 1998; Samelis et al., 2000). Furthermore, meat isolates of *Leuc. carnosum* have been shown to produce bacteriocins (van Laack et al., 1992; Felix et al., 1994; Keppler et al., 1994; Parente et al., 1996). Previously, it has been reported that the use of *Leuc. carnosum* in meat is not advisable due to its adverse effect on the sensory quality as well as its spoilage potential (van Laack et al., 1992). Björkroth et al. (1998) characterized *Leuc. carnosum* as the specific spoilage LAB of cooked, sliced, and vacuum-packed ham, and found that certain genotypes of this species were distributed throughout the ham processing line. Among the strains isolated from meat products and meat processing environments, none of the bacteriocin-producing *Leuc. carnosum* strains was found to be associated with spoilage (Björkroth et al., 1998). This shows that the spoilage potential of *Leuc. carnosum* differs depending on the strain. Based on the characterization of volatiles, *Leuc. carnosum* 4010 was grouped together with the noninoculated controls due to the high content of acetone and ethanol, which are often found in meat products (Hinrichsen and Andersen, 1994). The results strongly indicate that *Leuc. carnosum* 4010 did not produce any specific volatile compound that might affect the sensory quality of meat products, which was also verified by the sensory evaluation. In contrast, *C. piscicola* produced volatiles like 2- and 3-methylbutanal, 2-methylpropanal, 3-methylbutanol, and 2-methylpropanol, which may be products of amino acid catabolism (Hemme et al., 1982).

To serve as an additional safety factor in cooked, sliced, and vacuum-packed meat products, a pronounced antilisterial activity at low temperature is a prerequisite. *Leuc. carnosum* 4010 showed inhibitory activity against all *Listeria* strains examined and a variety of LAB strains as well. Also carnosin 44A produced by *Leuc. carnosum* LA44A inhibited all tested serotypes of *Listeria* spp. including five species and six serotypes (van Laack et al., 1992). Furthermore, *Leuc. carnosum* 4010 produced bacteriocin at low temperature (5 °C) in the meat product. The production of bacteriocin greatly depends on the growth temperature, and maximum bacteriocin production usually occurs at optimal growth temperature for the producer strain (Daba et al., 1993; Leroy and De Vuyst, 1999). Additional examinations of the production and identity of the bacteriocins showed that bacteriocin production was initiated during the exponential growth phase, reaching the highest level in the early stationary growth phase. Production of bacteriocins from lactic acid bacteria is often growth-associated with a maximum production in the exponential growth phase or at the beginning of the stationary phase (van Laack et al., 1992; Keppler et al., 1994; Enan et al., 1996; Todorov et al., 1999).

It was demonstrated that *Leuc. carnosum* 4010 produced two bacteriocins, namely leucocin A-4010 and leucocin B-4010. The N-terminal 30 residues showed that leucocin A-4010 was identical to leucocin A-UAL 187 produced by *Leuc. gelidum* UAL 187 (Hastings et al., 1991) and leucocin B-Ta11a produced by *Leuc. carnosum* Ta11a (Felix et al., 1994). The other bacteriocin, leucocin B-4010, was almost similar to the partial sequenced leucocin C-TA33 produced by *Leuc. mesenteroides* TA33a (Papathanasopoulos et al., 1998). Compared to leucocin C-TA33, alanine was replaced by a tryptophan at position 22 and additional three amino acids were identified for leucocin B-4010. Leucocin B-4010 was 100% identical to the partial sequenced leucocin 7C and leucocin 10C produced by *Leuc. mesenteroides*, which has been isolated from malted barley (Vaughan et al., 2001). Production of two bacteriocins from the same producer strain is well known and has among others also been reported for *Leuconostoc* strains (Revol

Junelles et al., 1996; Papathanasopoulos et al., 1997, 1998; Vaughan et al., 2001). For leucocin A-4010 and B-4010, further purification is required for separate analysis of the bacteriocins.

L. monocytogenes grew well on the cooked, sliced, and vacuum-packed meat sausage used in this experiment. This agrees with previous work dealing with the growth potential of this pathogen (Beumer et al., 1996; Bredholt et al., 1999). The present study showed that the kinetics of viability loss for *L. monocytogenes* greatly depended on the initial concentration of the bacteriocin-producing *Leuc. carnosum* 4010. Increased inoculation levels of *Leuc. carnosum* 4010 resulted in a more pronounced reduction in *L. monocytogenes*, which may be explained by production of a higher bacteriocin concentration at an earlier stage. Previously, it has been demonstrated that low initial numbers of *L. monocytogenes* ($10–10^3$ cfu/g) increased to $10^6–10^8$ cfu/g within the shelf life of cooked and vacuum-packed ham (30–37 days at 7–8 °C) even though high numbers of LAB were present at the end of storage (Beumer et al., 1996). Furthermore, addition of a nisin-producing *Lactococcus lactis* did not affect growth of *L. monocytogenes* in the product (Beumer et al., 1996). This demonstrates that the particular lactic acid bacteria chosen and the inoculation level are critical factors to obtain successful biopreservation.

5. Conclusion

This study showed that strains of *Leuc. carnosum* were found to be the predominant bacteriocin-producing bacteria in cooked, sliced, and vacuum-packed meat products. The characterization of the volatiles produced and the microbiological results demonstrated that the bacteriocin-producing strain, *Leuc. carnosum* 4010, can be used as protective culture for cold-stored, cooked, sliced, and vacuum-packed meat products. In order to enhance meat preservation by the use of *Leuc. carnosum* 4010, further efforts are currently in progress to determine how the interaction between *Leuc. carnosum* 4010 and *L. monocytogenes* is influenced by different meat additives and how protective cultures can be applied to sliced meat products.

Acknowledgements

The technical assistance of Heidi Grøn Asare, Gitte Rasmussen, Birgit Brunshuus Nielsen, Anne Marie Nielsen, Inge Lise Madsen, and Bente Isbye are gratefully acknowledged. The authors thank Kristian Rotvig Kristiansen, Department of Dairy and Food Science, The Royal Veterinary and Agricultural University, for helpful HPLC analyses. This work was financially supported by the Danish FØTEK 2 program (93 s-2469-å95-00064) and the Danish Bacon and Meat Council.

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