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Lactic acid bacteria isolated from rye sourdoughs produce bacteriocin-like inhibitory substances active against *Bacillus subtilis* and fungi

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Running head: Antimicrobial activity of rye sourdough LAB

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Abstract

Aim: To screen five strains of lactic acid bacteria (LAB) isolated from rye sourdoughs for the potential production of antimicrobial substances.

Methods and Results: *Lactobacillus sakei* KTU05-06, *Pediococcus acidilactici* KTU05-7, *P. pentosaceus* KTU05-8, KTU05-9 and KTU05-10 isolated from rye sourdoughs were investigated for production of bacteriocin-like inhibitory substances (BLIS). The supernatants of analysed LAB inhibited growth of up to 15 out of 25 indicator bacteria strains as well as up to 25 out of 56 LAB strains isolated from rye sourdoughs. Moreover these five LAB were active against ropes producing *B. subtilis* and the main bread mould spoilage causing fungi - *Aspergillus, Fusarium, Mucor* and *Penicillium*. *L. sakei* KTU05-6 demonstrated the best antibacterial properties and is resistant toward heat treatment even at 100^{o}C for 60 minutes.

Conclusions. The use of LAB producing antibacterial substances may be a good choice as a co-starter culture to ensure the stability of sourdoughs and to avoid the bacterial and fungi spoilage of the end-product.

Significance and Impact of the Study. The antimicrobial compounds designated as sakacin KTU05-6, pediocin KTU05-8 KTU05-9, KTU05-10 and AcKTU05-67 were not identical to any other known BLIS and this finding leads up to the assumption that they might be the novel.

K**eywords** Bacillus, Bacteriocins, Lactic acid bacteria, Fungi, Food safey

Introduction

During recent years, health-conscious consumers are looking for natural food which can fit into their healthy lifestyle. This includes food without additives as chemical preservatives, whereas the use of lactic acid bacteria and their antibacterial substances for biopreservation become more attractive to the food industry (Gálvez *et al.* 2007; Zotta *et al.* 2009). Sourdough is used as an essential ingredient for acidification, leavening and production of flavour compounds and biopreservation of bread (Katina *et al.* 2005; De Vuyst and Leroy 2007; Sadeghi 2008). In bakery practice, sourdough is usually sustained by repeated inoculation, whereby a reproducible and controlled composition and activity of the sourdough microflora is paramount to achieve a constant stability of sourdough as well as a high quality of the end-product (Rosenquist and Hansen 2000; Hammes *et al.* 1996).

The high stability of sourdoughs used for long periods might be caused by production of inhibitory substances by the sourdough microflora (Rosenquist and Hansen 2000; Messens and De Vuyst 2002). In addition, several researchers have reported about how sourdough bread can resist microbiological spoilage by moulds and rope-forming bacilli due to production of inhibitory substances (Katina *et al*. 2002; Hassan and Bullerman 2008; Sadehgi 2008; Valerio *et al*. 2009). Several sourdough lactic acid bacteria (LAB) produce inhibitory substances in varying degrees as organic acids (lactic acid, acetic acid), ethanol, diacetyl, hydrogen peroxide and carbon dioxide (Rosenquist and Hansen 1998). The inhibition, however, can also be caused by bacteriocins or bacteriocin-like inhibitory substances (BLIS). Bacteriocins are extracellularly released and ribosomally synthesized low molecule mass peptides, or proteins, with a bactericidal or bacteriostatic mode of action, in particular against a wide

range of mostly closely related Gram-positive bacteria (Klaenhammer 1993; Savadogo *et al*. 2006) and even against food-borne pathogens (Garneau *et al.* 2002), but the producer cells are immune to their own bacteriocins (De Vuyst and Leroy 2007). Thus, bacteriocins and other antimicrobial compounds in sourdoughs might be able to regulate the complex interactions within the starter microorganisms and the contaminant microflora and inhibit the growth of undesirable microorganisms like moulds and *Bacillus* (Hammes and Gänzle 1997, Rosenquist and Hansen 1998). Bacteriocins from LAB are described as "natural" inhibitors, in regard to LAB having a GRAS (generally regarded as safe) status (Guinane *et al*. 2005). Intensive research into the bacteriocins produced by LAB has been undertaken with the aim of improving the microbial quality and safety of fermented products (De Vuyst and Leroy 2007).

BLIS could be sensitive to heat treatment, pH, the activity of proteolytic enzymes and different storage conditions (Hoover and Chen 2005). Therefore, an important research goal is to study the activity of BLIS from rye sourdough LAB's by taking into acount their interaction with proteolytic enzymes and the affects of different factors.

In a previous study, LAB from rye sourdoughs were isolated and identified (Digaitiene *et al*. 2005). It is a matter of relevance to proceed with the study to characterize the potential production of BLIS from selected sourdough LAB. The objective of this study is therefore: to screen five strains of LAB isolated from rye sourdoughs for potential production of antimicrobial substances (i) to evaluate the antibacterial activity of the sourdough LAB against other strains of LAB as well as rope producing strains of *Bacillus* and bread mould spoilage causing fungus; (ii) to study the sensitivity of BLIS against proteinases, pH, heating and different storage conditions; (iii) to compare PCR fragments of the tested strains with PCR fragments of the structural genes in encoding for known bacteriocins.

Materials and methods

Sourdough lactic acid bacteria

The following strains *L. sakei* KTU05-6, *P. acidilactici* KTU05-7, *P. pentosaceus* KTU05-8, KTU05-9 and KTU05-10 were originally isolated from Lithuanian rye sourdoughs and selected due to their preliminary inhibiting properties; the LAB strains were phenotypic identified by growth in different media and genotypic identified by PCR amplification of 16S rDNA (Digaitiene *et al*. 2005). These strains were used for screening of potential production of BLIS. The LAB were propagated on modified MRS (mMRS) per liter: 10 g pepton, 10 g meat extract, 5 g yeast extract, 2 g sodium glyconate, 1 ml Tween 80, 2 g K_2HPO_4, 5 g sodium acetate, 2 g triammoniumcitrat, 0·2 g $MgSO_4 \cdot 7\ H_2O$, 0·05 g $MnSO_4 \cdot 4\ H_2O$, 0·5 g cystein-HCl, 15 g agar and 7 g each of glucose, fructose, maltose) at 30°C in a CO_2-atmosphere (Anaerocult A, Merck 13829).

Indicator strains of LAB used for testing of antimicrobial activity of sourdough LAB

Strains of LAB from different culture collections were used as sensitive indicator strains for testing of the antimicrobial activity of the five strains of sourdough LAB. The indicator strains were: *L. paracasei* NDC 0151 and BLG 17*, L. pontis* DSM 8475*, L. panis* DSM 6035, *L. buchneri* DSM 20057*, L. helveticus* IL 430*, P. acidilactici* PA-2 and PA-2-15*, Leuconostoc cremoris* Wis1200 and 1147*, L. cremoris* Wg2 and *Lact. lactis spp cremoris* MG1614*, Lact. lactis* IL1403 and *Streptococcus thermophilus* 3070

and S0. Additional nine other strains of LAB were used as indicator strains: *L. delbrüeckii* 76*, L. amylovorus* DSM 20531*, L. fermentum* DSH 20052*, L. sanfranciscensis* DSM 20451*, L. reuterii* DSM 20016*, L. plantarum* ATCC14917T and *L. brevis* DSM 20054 were obtained from the Department of Food Science, University of Copenhagen, Denmark, and *L. acidophilus* 336 and *L. bulgaricus* 140.3-148.3-3 from the Department of Food Technology, Kaunas University of Technology (Lithuania). The indicator strains were propagated in the following media: MRS for *Lactobacillus* and *Pediococcus*; LM17 for *Strep. thermophilus* S0; LM17 with 5% lactose for *Strep. thermophilus* 3070; GM17 with 1% glucose for *Lact. cremoris*; GM17 for *Lact. lactis ssp. cremoris* MG1614 and *Lact. lactis* IL1403.

Strains of LAB used for test of antimicrobial activity of the selected sourdough LAB

The antimicrobial activity of sourdough LAB was also tested against 56 indiginous strains of LAB representing nine different rye sourdoughs to investigate potential inhibition of other LAB. The strains of indigenous sourdough bacteria were: *P. pentosaceus* (22), *P. acidilactici* (10), *L. farciminis* (10), *L. curvatus* (9), *L. sakei* (1) and 4 non-identified *Lactobacillus*.

Rope producing strains of *B. subtilis* and bread mould spoilage causing fungus used for test of antimicrobial activity of sourdough LAB

Seven rope producing strains of *B. subtilis* (B1–B7) were obtained from Department of Veterinary Pathobiology, University of Copenhagen of Denmark (Rosenquist *et al*. 1998). They were used for testing of the sourdough LAB for antimicrobial effects

against *Bacillus.* The *Bacillus* strains were grown in BHI medium at 30°C. For preparation of spore suspension, 100 µl overnight cultures of vegetative strains of *Bacillus* were transferred to Petri dishes with modified Nutrient Agar (8 g nutrient broth, 8 g yeast extract, 0·01 g $MnCl_2$, 10 g agar agar per litre). After aerobic incubation for 3–5 days at 30°C, spores were harvested from the plate surfaces by washing twice with cold sterile distilled water and centrifuged at 1340 *g* for 20 min, resuspended in 10 ml sterile cold water and the suspension was stored at 4°C until use. Before use, the suspension was heated at 80°C for 10 min for heat activation of the spores and cooled to room temperature with cold water.

The same five LAB strains were tested to determinante their antifungal activities against *Aspergillus fumigatus, A. niger, A. versicolor, Penicillium chrysogenum, P. expansum* (obtained form Institute of Botany of Nature Research Centre, Laboratory of Biodeterioration Research), *F. culmorum* (obtained from Lithuanian Research Centre for Agriculture and Foresty, Institute of Agriculture), *A. flavus*, *F. poae*, *Mucor spp.* and *Penicillium spp* (isolated from contaminated wheat grain). The fungi were growing at 25°C on YEPD media (10 g yeast extract, 20 g glucose and peptone and 18 g agar) for 7 days. After growing fungi conidia were harvested from slants to prepare inoculums containing 1×10^4 asexual spores ml^{-1}.

Bacteriocin producing control strains of LAB

The control strains used for PCR detection of specific genes for bacteriocins produced by the sourdough LAB were: *Lact. lactis* CHCC4096 (nisin) and *P. acidilactici* PA-2 (pediocin PA) (C. Hansen A/S culture collection, Copenhagen, Denmark), *Lact. lactis* IFPL1 (lactacin 3147) (C. Palaez, Instituto del Frio-CSIC, Spain) (Martinez-Cuesta *et*

al. 2000) and *L. sakei* MI401 (sakacin P) (Department of Food Science, University of Copenhagen) (Larsen *et al*. 1993). *Lact. lactis* CHCC4096 and *Lact. lactis* IFPL105 were propagated in M17 with 1% glucose (GM17) at 30°C, *P. acidilactici* PA-2 and *L. sakei* MI401 were propagated in MRS at 30°C.

Determination of an antimicrobial activity of sourdough lactic acid bacteria

The antibacterial activity of LAB strains isolated from the Lithuanian rye sourdoughs was detected by the agar well diffusion assay by Shillinger and Lücke (1989). Petri dishes containing 1·5% agar and media specific for each indicator strain were overlaid with soft agar (0·7%) containing 100 µl of an overnight culture of the indicator strains. The five strains of LAB to be tested were grown at 30°C for 16 h in mMRS. Potential bacteriocin-like substances would be excreted into the medium during the growth phase and should occur in the supernatant fluids. 1·5 ml of the overnight cultures was centrifuged at 13000 *g* for 5 min. The supernatants were transferred to test tubes and stored at 4°C. Cell-free supernatants from each of the five strains were made by adjustment of the supernatants to pH 7.0 with 1 mol l^{-1} NaOH, heat-treatment for 3 min at 100°C, membrane filtered (0·2-µm), and filter-sterilized (0.45-µm) catalase (C1345, Sigma-Aldrich Co., Saint Louis, MO, USA) (2 mg ml^{-1}; 1600 U mg^{-1}) was added. The culture supernatants (50 µl) were added to each well (6 mm in diameter) punched in the agar plates. Prior to incubation for 24–48 hours at the optimal growth temperature, the plates were stored at 4°C for 4 hours to allow diffusion of compounds in the supernatant. Inhibition of bacteria was scored positive if the zone was wider than 1 mm. This method was used to test the antimicrobial activity of sourdough LAB against i) indicator strains of LAB; ii) sourdough lactic acid bacteria isolated from Lithianian

sourdoughs; iii) vegetative growth and spore germination of strains of rope producing *Bacillus* and bread spoilage causing fungi.

Data of antimicrobial activity against fungi were recorded daily on the second day of fungi incubation, according to the following scale: (−) no inhibition, (+/−) delay of conidia formation around the punched well was evaluated as fungistatic activity, (+) a very good inhibition of growthing of mycelium and conidia with large than 1 mm clear zones around the punched well was evaluated as fungicidal activity.

Sensitivity of BLIS to proteolytic enzymes, pH, heat treatment and different storage conditions

The sensitivity of the antimicrobial activities to different parameters was tested by pre-treatment of cell-free supernatant from growth of the sourdough LAB as described below followed by the determination of the residual activity by agar well diffusion assay. The most sensitive strain in the test for the antimicrobial activity of the five strains of sourdough LAB was selected as indicator strain. The following indicator strains were used: *Strep. thermophilus* 3070 for test of *L. sakei* KTU05-6; *L. acidophilus* 336 for test of *P. pentosaceus* KTU05-8 and KTU05-9; *L. bulgaricus* 140.3-148.3-3 for test of *P. pentosaceus* KTU05-10, and *Leuc. cremoris Wis* 1147 for test of *P. acidilactici* KTU05-7.

In order to determine the sensitivity to proteinases, 100 µl cell-free supernatant was pre-treated by incubation with 1 mg ml^{-1} proteinase K (70663, Merck KgaA, Darmstadt, Germany) for 30 min at 56°C. The pH stability was determined by re-adjusting the supernatants to pH 3·0, 3·5, 4·0, 8·0, 9·0 and 10 0 with 1 mol l^{-1} NaOH and 1 mol l^{-1} HCl, storing at 4°C for 4 hours followed by adjustment of the pH to 7·0. For

determination of the thermostability, the supernatants were heated at 100°C for 10, 20, 30 and 60 min followed by cooling. The storage stability was tested every 10 days by evaluating the activity of supernatants stored at –20°C, 4°C and 30°C for 50 days.

Kinetics of BLIS production

Production of the antimicrobial substances by the five strains of sourdough LAB was determined at different phases of growth (exponential and stationary) by inoculation of 1% of an overnight culture of the selected inhibited strains in mMRS broth at 30°C. Samples of 1·5 ml were withdrawn after 6, 10, 17, 24 and 30 hours of growth. The samples were assayed for antimicrobial activity by the agar well diffusion assay as mentioned above.

PCR amplification of genes encoding for known bacteriocins

Primers within the genes encoding for production of the specific bacteriocins nisin, pediocin PA, lacticin 3147 and sakacin P were designed and tested by PCR amplification of 16 S rDNA followed by sequencing in order to determine whether any of the genes could be found in the five sourdough LAB. The primers and annealing temperatures are listed in Table 2. A single colony was transferred to 200 µl sterile water containing 100 U mutanolysin followed by incubation at 37°C for 2 hours. Amplification mixtures consisted of 2 µl of mutanolysin-treated cell suspension, 10 µl 10*PCR-buffer (200 mmol l^{-1} Tris-HCl (pH 8·4), 500 mmol l^{-1} KCl), 5 U Taq polymerase, 2 mmol l^{-1} $MgCl_2$, 1 µl Formamide, 1 µmol l^{-1} of each primer and 200 µM dATP, dCTP, dGTP, dTTP in a final volume of 100 µl. The samples were amplified in a

PCR machine Robo Cycler Gradient 96 (Stratagene, California, USA). For detection of the structural genes encoding for the bacteriocins nisin, lacticin 3147, sakacin P and pediocin PA, the PCR amplification was carried out as follows: 5 min at 95°C; 30 cycles each consisting of 30 seconds at 95°C, 30 seconds at annealing temperature (48–60°C) and 30 seconds at 72°C. Amplified fragments were visualized on 1% agarose gel and were purified with GFX columns (Sigma-Aldrich Chemie GmbH, Munich, Germany) according to the manufacturer's instructions (Rodriguez, 2000). The amplified PCR products were directly sequenced in Beckman CEQ 2000XL (Beckman Coulter Inc., Fullerton, CA, USA). As marker, a mixture of 100 bp DNR GeneRuler (0.5 mg ml^{-1}) with colorant has been used.

Statistical analyses

All the experiments were carried out at least in three independent experiments. The means and standard deviations of the data were calculated.

Results

Screening of sourdough LAB for production of antimicrobial compounds against indicator strains

The inhibitory spectra of the five strains of sourdough LAB tested against 25 indicator strains of LAB to investigate their general inhibitory capacity against other LAB are shown in Table 1. The all supernatants of the five strains of analysed LAB showed different antimicrobial activity towards 15 out of 25 LAB strains belonging to 13 different species. The growth of two indicator strains (*L. bulgaricus* 140.3-148.3-3 and *P. acidilactici* PA-2-15) was inhibited by the supernatant of all five strains, while ten

indicator strains were not sensitive at all. One supernatant (from *L. sakei* KTU05-6) inhibited the growth of nine different species, while the other strains inhibited between five and seven strains, indicating that inhibitory substance from the five selected strains are indeed different from each other and they all have rather broad antimicrobial spectra against other LAB.

Screening of sourdough LAB for production of BLIS

The antimicrobial activity of the five strains of LAB was also tested against 56 strains of LAB isolated from nine different rye sourdoughs to investigate potential inhibition of sourdough LAB. The results showed that the analysed LAB demonstrated different antimicrobial activity against the strains of LAB isolated from indigenous rye sourdoughs (Table 2).

P. acidilactici KTU05-7 and *P. pentosaceus* KTU05-10 were both isolated from sourdough F. Their inhition spectra were quite different as *P. acidilactici* KTU05-7 inhibited resident LAB strains (25/56) both *Lactobacillus* and *Pediococcus* from all the other sourdoughs except sourdough F, while *P. pentosaceus* KTU05-10 mainly inhibited strains from sourdough H (*Lactobacillus*). *L. sakei* KTU05-6 was isolated from sourdough K and it was able to inhibit the growth 6/8 strains from sourdough H and even 3/12 strains from sourdough K where it was isolated from, while the inhibition of the resident LAB from the other sourdoughs was less. Inhibition of some strains of sourdough LAB by other LAB isolated from sourdoughs might increase the microbial stability of the sourdoughs as the inhibiting strains can dominate the other strains.

Sensibility of BLIS to proteinase K, pH, storage and heat treatment

The stability of the antimicrobial activity of sakacin 05-6, pediocin 05-8, pediocin 05-9, pediocin 05-10 and pediocin Ac05-7 was evaluated under influence of proteinase K, pH, storage and heat treatment (Table 3). All five bacteriocin-like substances were found to be sensitive to proteolytic enzymes, as the activity was totally destroyed after incubation with 1 mg ml^{-1} proteinase K for 30 min. This indicates that they might be bacteriocins as protease sensitivity is a key criterion in the characterization of antimicrobial substances as bacteriocins (Klaenhammer 1993).

The influence of pH on the antimicrobial activity was evaluated in the pH range 3·0–10·0. All BLIS had activity in the pH-range for doughs and sourdoughs (pH 3·5–6·0). Sakacin 05-6 had antimicrobial activity in the pH range 3·0–8·0 but was sensitive to alkaline treatment. Pediocin 05-8 and pediocin 05-9 were active in pH range 3·5–10·0, pediocin 05-10 in the pH range 3·5–8·0, and pediocin Ac05-7 was active in the pH range 3·5–9·0. The optimum activity was in pH range 7·0–8·0 for the pediocins while no activity was seen at pH 3·0.

The activity of sakacin 05-6, pediocin 05-8 and pediocin 05-9 was stable during storage at –20°C and at +4°C temperature for at least 50 days. Pediocin 05-10 and pediocin Ac05-7 were also active after 50 days of storage at 30°C, while sakacin 05-6, pediocin 05-8 and pediocin 05-9 had lost their activity under this storage condition.

The activity of bacteriocin-like substances after heating was dependent on the producer strain. Sakacin 05-6 and pediocin Ac05-7 appeared to be stable even after treatment at 100°C for 60 min, while the activity of pediocin 05-9 and pediocin 05-10 was totally lost after treatment at 100°C for 30 min. Pediocin 05-8 was very sensitive to heat treatment and was inactivated after heating for 20 min.

The maximum activity of BLIS produced by the five sourdough strains, as evaluated by well diffusion assay against corresponding indicator strains, was obtained after 17–24 hours of incubation except for pediocins 05-8 and 05-10, which had maximum activity after 10–17 hours (Fig. 1). The inhibitory activity of sakacin 05-6, pediocin 05-9, pediocin 05-10 and pediocin Ac05-7 was detected after 6 hours of incubation when the producer organisms were in the early exponential growth phase, while pediocin 05-8 was detected after 10 hours of incubation. After 30 hours of incubation, the activity of sakacin 05-6, pediocin 05-9 and pediocin Ac05-7 was lower, while the activity of pediocin 05-8 and pediocin 05-10 was totally lost.

Structural genes encoding known bacteriocins

Primers within the structural genes encoding for production of lacticin 3147 (P1), pediocin PA (P2), nisin (P3) and sakacin P (P4) (Table 4) were designed in order to determine whether any of the genes could be found in the five sourdough LAB. Visualization of the amplified PCR fragments of the four tested bacteriocins is shown in Fig. 2. When primer set P1 was used for PCR amplification with chromosomal DNA of the control strain *Lactococcus lactis* IFPL105 as template (Fig. 2A), a PCR fragment of 257 bp (lane A6) was observed. When the five sourdough strains *L. sakei* KTU05-6 (1), *P. acidilactici* KTU05-7 (2), *P. pentosaceus* KTU05-8 (3), KTU05-9 (4) and KTU05-10 (5) were used as template, a weak PCR product of a similar size was observed for *L. sakei* KTU05-6 (lane A1) and *P. pentosaceus* KTU05-10 (lane A5), while the PCR products from *P. acidilactici* KTU05-7 (lane A2) and *P. pentosaceus* KTU05-8 (lane A3) were much larger than the expected 257 bp. When primer sets P2 for production of pediocin PA (Fig. 2B), P3 for production of nisin (Fig. 2C), and P4 for production of

sakacin P (Fig. 2D) were used on tested strains, only the control strains for production of the known bacteriocins had PCR fragments of the correct size: 541 bp in lane P2 for control strain *P. acidilactici* (Fig. 2B), 590 bp in lane P3 for control strain *Lact. lactis* CHCC4096 (Figure 2C), and 150 bp in lane P4 for the control strain *L. sakei* MI401 (Fig. 2D).

This showed that none of the strains was identical as they all gave different patterns of PCR products obtained with primer sets 1–4, and none of them contained genes encoding for pediocin PA, nisin or sakacin P. Only *L. sakei* KTU05-6 (lane A1) and *P. pentosaceus* KTU05-10 (lane A5) in Fig. 2A had weak bands of the same size as the control strain for production of lactacin 3147. However, analysis of the inhibition spectra of the antimicrobial substances from the two sourdough LAB showed that sakacin 05-6 and pediocin 05-10 have different inhibition spectra (Tables 1, 3 and 6), and they might not be identical to lacticin 3147 as their inhibition specters differ from this (Table 5).

Antimicrobial effect of sourdough LAB against rope producing strains of *Bacillus* and bread spoilage causing fungi

The ability of the supernatants of the five sourdough LAB to inhibit the growth of vegetative cells after spore activation of rope producing *Bacillus* was tested against seven strains of *B. subtilis* isolated from bread products. None of the LAB strains was able to inhibit the growth of the vegetative *Bacillus* cells. However, the supernatants of five sourdough LAB were able to inhibite growth of activated spores from four out of seven strains of *B. subtillis* in varying degree (Table 6). Furthermore, all five sourdough LAB produced BLIS show fungicidal activity (observed a very good inhibition of mycelium and conidia growthing with large than 1 mm clear zones around the punched

well) against *Fusarium culmorum*. *L.sakei* KTU05-6, *P. acidilactici* KTU05-7 and *P. pentosaceus* KTU05-9 inhibit the growth of *Aspergillus flavus*, *Fusarium poae*, *Mucor* spp. (fungicidal activity). *L.sakei* KTU05-6 and *P. acidilactici* KTU05-7 destroyed the growth of *Penicillium* spp. isolated from contaminated wheat grain. All five tested sourdough LAB delayed the conidia formations around the punched well of *Aspergillus niger* (fungistatic avtivity) until fourth days, whereas the conidia formations of *Aspergillus fumigatus* inhibit *P. pentosaceus* KTU05-8 and KTU05-10 while *Aspergillus versicolor*, *Penicilium expansum* and *P. crysogenum* the conidias formations were suppressed by four out of five tested sourdough LAB (Table 6).

Discussion

All tested sourdough LAB (*Lactobacillus sakei* KTU05-6, *Pediococcus acidilactici* KTU05-7, *P. pentosaceus* KTU05-8, *P. pentosaceus* KTU05-9 and *P. pentosaceus* KTU05-10) showed antibacterial activity against other LAB, a characteristic which has also been reported for other antimicrobial substances produced by sourdough LAB, such as BLIS C57, reutericyclin, sakacin P (previous bavaricin A) and plantaricin ST31 (Larsen *et al*. 1993; Gänzle *et al*. 2000; Corsetti *et al*. 2004).

The potential to use BLIS-producing LAB as starter cultures depends on their ability to inhibit other microorganisms present in sourdoughs. The use of bacteriocins or strain of bacteriocin-producing sourdough LAB that are active against *Bacillus subtilis* species can be advantageous, since *Bacillus* may cause spoilage of bread and may constitute a health risk (Rosenquist and Hansen 1998). Tested *L. sakei* KTU05-6, *P. acidilactici* KTU05-7, *P. pentosaceus* KTU05-8, *P. pentosaceus* KTU05-9 and *P. pentosaceus* KTU05-10 were able to inhibite growth of activated spores of *B. subtillis* in varying

degree, furthermore show wide spectrum of antimicrobial activity against *A. flavus*, *A.fumigatus*, *A. niger*, *A. versicolor*, *F. culmorum*, *F. poae*, *Mucor spp*., *P. chrysogenum*, *P. expansum* and *Penicillium spp*., which are most popular contaminants from grains. This indicates that the strains or their inhibitory substance might have a potential use as anti-ropiness and anti-fungal agents in the bread industry.

The antimicrobial compounds produced by the *L. sakei* KTU05-6, *P. pentosaceus* KTU05-8, *P. pentosaceus* KTU05-9, *P. pentosaceus* KTU05-10 and *P. acidilactici* KTU05-7 were designated as sakacin 05-6, pediocin 05-8, pediocin 05-9, pediocin 05-10 and pediocin Ac05-7, respectively. The antimicrobial substances investigated in this study are referred to as BLIS as they have not been isolated and their amino acid sequences have not been characterized.

Since the *Bacillus* spores are resistant to heat and some of them can survive the baking process (Bailey and von Holy 1993; Röcken and Voysey 1995), it is also important that bacteriocins can resist the temperature present during the baking process where the temperature of the bread crumb reaches 98^{o}C. Thus, if the bacteriocin is present in the final bread, it may inhibit spore germination and in this way eliminate or reduce ropiness of bread. In our study it was found that sakacin 806 and pediocin Ac807 might be active in bread crumb even after baking, hence, if the conditions prevailing in sourdough can support the growth of *L. sakei* KTU05-6, this strain might be used to control rope formation in bread as it has the most inhibiting effect against *B. subtilis*.

Leroy and De Vuyst (1999) reported the highest activity by sakacin K to be at pH 5·0, rather low activity at pH 5·5 and none activity at pH 4·5, meaning that the pH range for a good sakacin production is very narrow, whereasin in our studies examined sakacin 05-6 had antimicrobial activity in the pH range 3·0–8·0. However, analysis of the inhibition spectra of the antimicrobial substances from the two sourdough LAB showed

that sakacin 05-6 and pediocin 05-10 have different inhibition spectra, and they might not be identical to lacticin 3147 as their inhibition specters differ from this. Other investigations have shown that the growth of *B. subtilis* strains in culture medium could be inhibited by three different compounds isolated from sourdoughs: BLIS C57, reutericyclin and plantaricin ST31 (Gänzle *et al*. 2000). The bacteriocin plantaricin ST31 is the most heat-resistant compound, whereas the activity of reutericyclin could be lost at baking temperatures, and the heat resistance of BLIS C57 has not been determined. In bakery practice sourdoughs usually are used for bread production by a final pH of 3·5–3·8 which is reached within 12–24 hours (Hansen 2004), so the production of BLIS is highest at the time when the sourdough is to be used in bread production and for propagation of the unfermented sourdough for the next day´s bread production (exponential to stationary growth phase). This might be important for the microbial stability of industrial sourdoughs as reported by Rosenquist and Hansen (2000) and Messens and De Vuyst (2002).

Quite a few studies on a bacterocin activity possessed by *L. sakei* strains have been performed and can be compared with antimicrobial activity of other BLIS. Schillinger and Lucke (1989) reported about 221 surveyed lactobacilli strains, among those 19 strains of *L. sakei*, three strains of *L. plantarum* and one strain of *L. curvatus* which were found to inhibit other lactobacilli. The bacteriocins were not identical according to the evaluation of supernatant's antimicrobial spectra as sakacin A produced by *L. sakei* Lb706 was active against *Listeria monocytogenes* strains 8732 and 17a, moreover four other strains of *L. sakei* and one strain of *L. plantarum* also showed antilisterial activity. Mørtvedt and Nes (1990) identified bacteriocin Lactosin S produced by *L. sakei* and described it as a moderate heat stable, sensitive to protease and having antimicrobial activity against *Lactobacillus, Pediococcus,* and *Leuconostoc* genera members.

According to literature anti-fungal activity during fermentation process produce organic acids and other small molecular weight compounds, but no one studies exactly confirm that anti-fungal activity show bacteriocins (Schillinger and Villarreal 2010).

Conclusions

L. sakei KTU05-6, *P. acidilactici* KTU05-7, *P. pentosaceus* KTU05-8, KTU05-9 and KTU05-10 isolated from the rye sourdougs able to produce not identical to any other known BLIS were characterized as to have a good antimicrobial properties against *B. subtilis* and some fungi. The use of these sourdough LAB with antimicrobial activity as starter for bread making could be a good alternative to ensure the stability of sourdoughs and to obtain more safe bread products.

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Table 1 Inhibitory activity of tested LAB against indicator strains of LAB

Indicator strains	Sourdough LAB				
	KTU05-6	KTU05-8	KTU05-9	KTU05-10	KTU05-7
Lactobacillus bulgaricus 140.3-148.3-3	2·50±0·00*	2·00±0·00	1·75±0·35	2·25±0·35	1·90±0·14
L. acidophilus 336	2·90±0·14	2·75±0·35	4·00±0·00	2·50±0·00	–
L. delbrueckii 76	–	–	–	–	–
L. paracasei NCD 0151	–	–	–	–	–
L. paracasei BLG 17	–	–	2·00±0·00	–	–
L. pontis DSM 8475	1·00±0·00	–	–	2·50±0·00	–
L. panis DSM 6035	–	–	–	2·25±0·35	–
L. buchneri DSM 20057	–	–	–	1·00±0·00	–
L. helveticus IL 430	–	–	–	–	–
L. sanfranciscensis DSM 20451	1·25±0·35	–	2·75±0·35	–	–
L. amylovorus DSM 20531	–	–	–	–	–
L. plantarum ATCC14917T	–	–	–	–	–
L. fermentum DSH 20052	2·00±0·00	1·25±0·35	–	–	–
L. reuterii DSM 20016	1·50±0·00	–	1·75±0·35	–	2·00±0·00
L. brevis DSM 20054	–	–	–	–	–
Pediococcus acidilactici DSM 20284	–	–	–	–	–
P. acidilactici PA-2	–	1·50±0·00	2·50±0·00	–	–
P. acidilactici PA-2-15	1·00±0·00	2·00±0·00	1·25±0·35	1·25±0·35	1·75±0·35
Leuconostoc cremoris Wis 1200	–	–	–	–	–
Leuc. cremoris Wis 1147	–	2·50±0·00	1·00±0·00	–	3·50±0·00
Streptococcus thermophilus 3070	4·50±0·00	–	–	–	1·50±0·00
Strep. thermophilus S0	1·25±0·35	1·50±0·00	–	–	–
Lactococcus cremoris Wg 2	1·00±0·00	1·25±0·35	–	–	2·25±0·35
Lactococcus lactis ssp.cremoris MG1614	–	–	–	–	–
L. lactis IL1403	–	–	–	–	–

* Width of the inhibition zone, (mm). '–', No inhibition zone. Results indicate mean ± SD of three independent experiments.

Table 2 Antibacterial activity of tested LAB against indigenous LAB isolated from rye sourdoughs

Sourdough	Ratio of indigenous LAB in sourdough	Number of isolates tested	Number of sensitive isolates from rye sourdoughs				
			L. sakei KTU05-6	*P. acidilactici* KTU05-7	*P. pentosaceus* KTU05-8	*P. pentosaceus* KTU05-9	*P. pentosaceus* KTU05-10
A	100% *P. pentosaceus*	3	1	2	–	–	–
B	100% *P. pentosaceus*	4	–	4	–	3	–
C	100% *P. pentosaceus*	6	–	5	–	–	–
D	71% *P. pentosaceus*	5	1	4	–	1	–
	29% *P. acidilactici*	2	1	2	1	–	
E	25% *P. pentosaceus*	2	1	1	1	1	1
	75% *P. acidilactici*	6	–	2	–	1	–
F	50% *P. pentosaceus*	2	–	–	–	–	–
	50% *P. acidilactici*	2					
G	100% *L .farciminis*	4	1	–	2	1	1
H	50% *L. farciminis*	4	4	2	4	4	3
	25% *L. curvatus*	2	1	1	2	2	1
	25% *Lactobacillus spp.*	2	1	–	2	2	1
K	17% *L. farciminis*	2	1	1	–	1	–
	8% *L. sakei*	1	–	–	–	–	–
	58% *Lactobacillus spp.*	2	2	1	2	4	1
	17% *L. curvatus*	7	–	–	1	–	–

'–', No inhibition zone.

Table 3 Effect of proteinase K, pH, storage and heat treatment on antimicrobial activity of sakacin 806, pediocin 808, pediocin 809, pediocin 810 and pediocin Ac807

Treatment	Bacteriocins				
	[a]sakacin 05-6	[b]pediocin 05-8	[c]pediocin 05-9	[d]pediocin 05-10	[e]pediocin Ac05-7
Control (not treated)	4·50±0·00*	2·75±0·35	4·00±0·00	2·25±0·35	3·50±0·00
Proteinase K	–	–	–	–	–
pH					
3	2·35±0·21	–	–	–	–
3·5	3·00±0·00	1·75±0·35	1·80±0·28	1·75±0·35	1·75±0·35
4	3·50±0·00	1·85±0·49	1·75±0·35	1·80±0·42	1·75±0·35
7	4·50±0·00	2·75±0·35	4·00±0·00	2·25±0·35	3·50±0·00
8	2·00±0·00	2·50±0·00	2·00±0·00	1·35±0·21	2·25±0·35
9	–	2·75±0·35	1·75±0·35	–	1·25±0·35
10	–	1·50±0·00	1·25±0·35	–	–
Storage for 50 days					
–20°C	3·00±0·00	1·50±0·00	2·00±0·00	1·50±0·00	2·65±0·21
+4°C	3·00±0·00	2·00±0·00	2·25±0·35	2·00±0·00	2·50±0·00
+30°C	– (after 30 days)	– (after 30	– (after 40	1·00±0·00	1·50±0·00
Heat treatment		days)	days)		
100^0C for 10 min	4·00±0·00			1·00±0·00	2·25±0·35
100^0C for 20 min	4·00±0·00	2·00±0·00	2·00±0·00	1·00±0·00	2·.00±0·00
100^0C for 30 min	3·75±0·35	–	1·50±0·00	–	1·.05±0·.00
100^0C for 60 min	3·75±0·35	–	–	–	1·75±0·35

* Width of the inhibition zone, (mm). "–", No inhibition zone. Results indicate mean ± SD. of three independent experiments. Indicator strains: [a] *Strep. thermophilus* 3070, [b, c] *Lb. acidophilus* 336, [d] *Lb. bulgaricus* 140.3-148.3-3, [e] *Leuc. cremoris Wis* 1147.

Table 4 Oligonucleotide primers used for the detection of genes encoding known bacteriocins

Primer	Specifity	5'-sequence-3'	Size, bp	t, °C
P1	Lacticin 3147	TGAAGATGTATTTGGTGCGT	257	60
		CAGGAGTTGCTGGTGTTGTT		60
P2	Pediocin PA	GCGCCTGCAGGGCTTTCTTTCGATCACGAT	541	52
		GCGCGTCGACGGTTCGATAGTATCGTGCTT		52
P3	Nisin	CTATGTACACCCGGTTGTAA	590	48
		TTTATGAACTAGGCGAATCA		48
P4	Sakacin P	ACAGGTGGAAAATATTATGGTA	150	48
		TTTTGCTTATTATTTATTCCAG		48

Table 5 Comparison of the inhibition spectra of lacticin 3147* and sakacin 05-6 and pediocin 05-10

Indicator strain in literature	Lacticin 3147	Sakacin 05-6	Pediocin 05-10
L. reuteri DSM20016	+	±	–
Strep. termophilus SO	+	±	–
L. plantarum ATCC14917T	+	–	–
Lactococcus. lactis subsp. cremoris G1614	+	–	–

* *Lactococcus. lactis* IFLP105 was used. '+' With inhibition. '–' No inhibition zone

Table 6 Inhibition of spore germination from *B. subtilis* and fungi by bacteriocins of LAB isolated from rye sourdoughs

Microorganism		Source	Sourdough LAB				
			KTU05-6	KTU05-8	KTU05-9	KTU05-10	KTU05-7
Bacillus subtilis strain	B1	White bread I	1·90±0·14*	–	2·00±0·00	1·00±0·00	1·35±0·21
	B2	Ropy wholemeal bread II	2·00±0·00	–	–	–	–
	B3	Ropy wholemeal bread III	–	–	–	–	–
	B4	Ropy wholemeal bread IV	–	–	–	–	–
	B5	Wholemeal bread V	1·65±0·21	–	–	–	–
	B6	Wholemeal bread VI	–	1·00±0·00	1·50±0·00	2·00±0·00	1·25±0·35
	B7	Malted rye bread powder VII	–	–	–	–	–
Aspergillus flavus		maize	+	ND	+	ND	+
Aspergillus fumigatus		wheat	–	+/–	–	+/–	–
Aspergillus niger		wheat	+/–	+/–	+/–	+/–	+/–
Aspergillus versicolor		wheat	+/–	+/–	–	+/–	+/–
Fusarium culmorum		maize	+	+	+	+	+
Fusarium poae		maize	+	ND	+	ND	+
Mucor spp.		maize	+	ND	+	ND	+
Penicillium chrysogenum		wheat	–	+/–	+/–	+/–	+/–
Penicillium expansum		wheat	+/–	+/–	+/–	–	+/–
Penicillium spp.		maize	+	ND	–	ND	+

* Width of the inhibition zone, (mm). '–', No inhibition zone. Results indicate mean ± SD of three independent experiments.

Fungi infibition evaluated according to the following scale: (–) no inhibition, (+/–) delay of conidia formation around the punched well was evaluated as fungistatic activity, (+) a very good inhibition of growthing of mycelium and conidia with large than 1 mm clear zones around the punched well was evaluated as fungicidal activity; (ND) not determined.

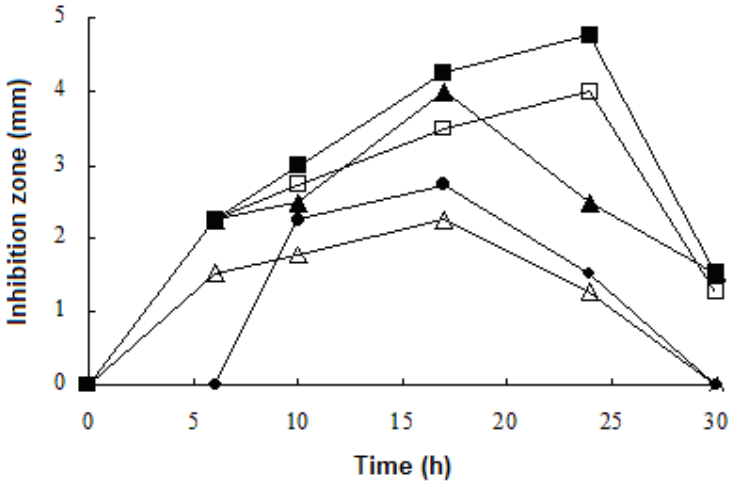

Figure 1

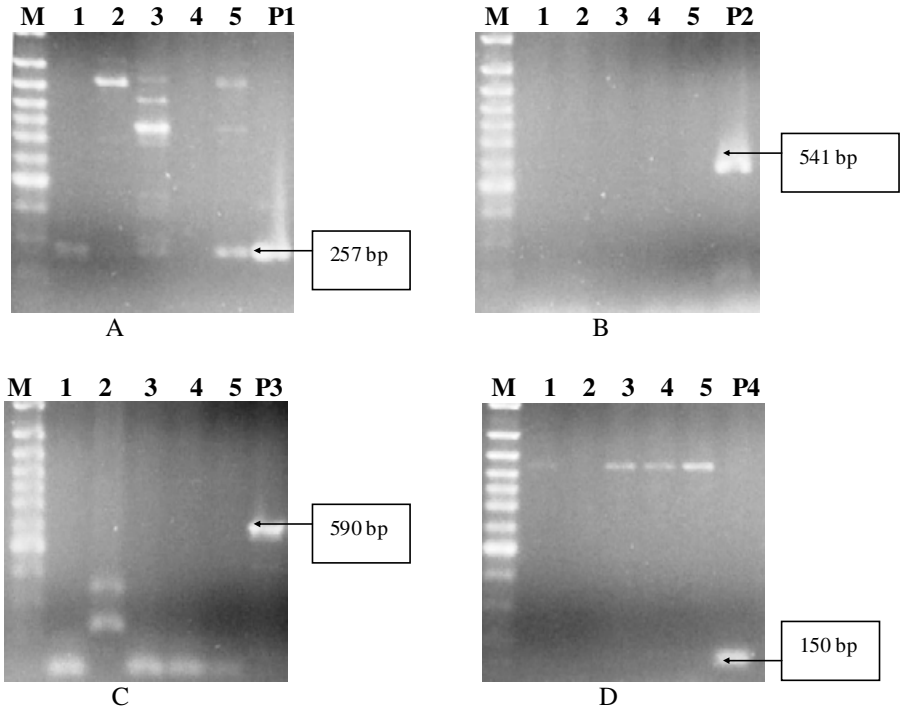

Figure 2

Figure legends

Fig. 1 Production of BLIS by sourdough LAB: ■ sakacin 05-6; ● pediocin 05-8; ▲ pediocin 05-9; Δ pediocin 05-10; □ pediocin Ac05-7 during incubation (the points are an average of three independent experiments).

Figure 2 Gel electrophoresis of the PCR fragments amplified with primer set for P1 (A), P2 (B), P3 (C) and P4 (D). M – marker Gene Ruler 100-bp DNA Ladder plus; templates used for PCR amplification are genomic DNA from *L. sakei* KTU05-6 (lane 1), *P. acidilactici* KTU05-7 (lane 2); *P. pentosaceus* KTU05-8 (lane 3), *P. pentosaceus* KTU05-9 (lane 4), *P. pentosaceus* KTU05-10 (lane 5), *Lact. lactis* IFPL 1 (lane P1), *P. acidilactici* PA-2 (lane P2), *Lact. lactis* CHCC4096 (lane P3), *L. sakei* MI 401 (lane P4).