An investigation of the bacteriocinogenic potential of lactic acid bacteria associated with wheat (*Triticum durum*) kernels and non-conventional flours

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Abstract

One hundred and thirty-seven lactic acid bacteria (LAB), previously isolated from wheat (*Triticum durum*) grains and non-conventional flour samples, were tested for the production of antibacterial substances. A total of 16 strains (5 *Enterococcus faecium*, 5 *Enterococcus mundtii*, 4 *Pediococcus pentosaceus*, 1 *Lactobacillus coryniformis* and 1 *Lactococcus garvieae*) were found to inhibit the growth of *Listeria innocua*. The antibacterial activities were preliminarily investigated for their general behaviour with proteolytic (proteinase K, protease B and trypsin), amylolytic (α-amylase) and lipolytic (lipase) enzymes, after heat treatment, and exposure to different pHs and ethanol concentrations. Bacteriocin-like inhibitory substances (BLIS) were also characterized for their inhibition spectra against non-pathogenic and pathogenic food-associated and human pathogenic bacteria. LAB showing the best characteristics in terms of inhibition spectrum, inhibition activity and mode of action (bactericidal) belonged to the species *Ent. mundtii*. The high percentage (11.68%) of BLIS-producing strains detected confirmed previous observations that raw materials may harbour higher numbers of bacteriocinogenic LAB than fermented foods.

Introduction

Bacteriocins are ribosomally synthesized, extracellularly released low-molecular-mass peptides or proteins which have a bactericidal or bacteriostatic effect on other (usually closed related) species (Chen & Hoover, 2003; Cotter, Hill, & Ross, 2005). Bacteriocin production has been found in numerous species of bacteria, among which, due to their "Generally Recognized As Safe" (GRAS) status, bacteriocins produced by lactic acid bacteria (LAB) have attracted great interest in terms of food safety (Cleveland, Montville, Nes, & Chikindas, 2001; Eijsink et al., 2002; Garneau, Martin, & Vederas, 2002). De Vuyst and Vandamme reported in 1994 that the majority of bacteriocins described at the time of writing were those produced by *Lactobacillus* spp., followed by *Enterococcus*, *Pediococcus* and *Leuconostoc* spp.; nowadays, other bacteriocins from the group of LAB, such as those produced by *Lactococcus*, *Streptococcus* and *Carnobacterium* have been described, but so far, the only bacteriocin approved for utilization as a preservative in many foods by the US Food and Drug Administration is nisin (Federal Register, 1988).

Regarding the application of bacteriocin-producing starters in food fermentation, the major problem is related

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to the *in situ* antimicrobial efficacy which can be negatively influenced by various factors, such as binding of the bacteriocins to food components, inactivation by proteases, changes in solubility and charge, changes in the cell envelope of the target bacteria (Aesen et al., 2003; Gänzle, Weber, & Hammes, 1999). Furthermore, unlike their general behaviour represented by being active against closely related species, LAB bacteriocins have also been found to be active against Gram-negative bacteria (Abriouel, Valdivia, Gálvez, & Maqueda, 2001; Caridi, 2002; Ko & Ahn, 2000; Messi, Bondi, Sabia, Battini, & Manicardi, 2001; Torodov & Dicks, 2004a, 2005), hence the interest in their application is also forwarded to the gastro intestinal tract. The interaction between LAB bacteriocins and Gram-negative bacteria is not clearly understood, e.g. Kuwano et al. (2005) suggested that nisin Z utilizes a high-salt-sensitive mechanism of antibacterial activity for *Escherichia coli*.

Pratical observation (Van Sinderen, oral communication) revealed that LAB isolated from raw materials show a higher ability, in terms of number of positive strains and inhibition activity, to produce bacteriocins than LAB associated with fermented foods where the adaptation to the particular environment (e.g. to nutritional sources) seems to play a major role for their persistence. To our knowledge, this is the first work dealing with the investigation of BLIS production from LAB associated with wheat kernels and non-conventional flours.

The present study is part of a project aimed at characterizing the LAB isolated from sourdough raw materials (see also Corsetti et al., in press) for their potential use in food fermentation and biopreservation. Based on the above considerations, the aims of this study were to screen 137 LAB strains for their antibacterial potential and preliminarily characterize the active compounds.

Materials and methods

Strains and growth conditions

Bacterial strains used as indicators for bacteriocin assays are listed in Table 1. *Listeria innocua* 4202 (belonging to the culture collection of National Food Biotechnology Centre, University College Cork, Ireland) was propagated in BHI (Oxoid, Milan, Italy) at 37 °C for 24 h, *Lactobacillus sakei* LMG 2313 (obtained from the Laboratory of Microbial Gene Technology, Ås, Norway) in modified-MRS (mMRS) (maltose and fresh yeast extract were added at final concentrations of 1% and 10%, respectively, and the final pH was adjusted to 5.6) at 30 °C for 24 h, while all other strains were propagated as indicated by the respective culture collection. Strains to be tested for antibacterial compound production, previously isolated from wheat grain samples and different flours used in bread-making (Corsetti et al., in press), were propagated in the corresponding medium used for isolation: Sour Dough Bacteria (SDB) (Kline & Sugihara, 1971), San Francisco Medium (SFM) (Vogel et al., 1994), MRS (Oxoid) and M17 (Oxoid).

Assays for bacteriocin activity

The antimicrobial activity of LAB was first detected by the agar-spot deferred method and the strains showing positive results were subsequently tested by the well diffusion assay (Schillinger & Lücke, 1989). Both assays were performed following the modifications of Corsetti, Settanni, and Van Sinderen (2004) using *L. innocua* 4202 and *Lb. sakei* LMG 2313 as indicator strains.

The antibacterial activity of the supernatants was evaluated by the critical dilution assay of Barefoot and Klaenhammer (1983). Bacteriocin activity was defined as the reciprocal of the highest dilution showing definite inhibition of the indicator strains and was expressed as activity units per millilitre (AU/ml).

Effect of enzymes, heat treatment, pH and organic solvent on the antimicrobial activity

Sensitivity of each active supernatant (AS) to enzymes was tested as reported by Corsetti et al. (2004) by well diffusion assay.

Sensitivity to high temperatures was determined by heat treatment of the ASs at 100 °C for 20 min, 100 °C for 60 min and by sterilization (121 °C for 15 min). The ASs were adjusted to varying pH values, ranging between 3.0 and 11.0, with 5 M HCl or 5 M NaOH, incubated at 28 °C for 1 h and assayed for residual activity. The effect of organic solvents was evaluated after concentration of the ASs under vacuum (SC110A SpeedVac® Plus, Thermo Savant, Rome, Italy) and subsequent addition of ethanol (5%, 10%, and 15%) and water to bring ASs to the initial volume.

Cross-sensitivity test

Strains positive for production of protein antimicrobial compounds were challenged by each other to test for cross sensitivity. Bacteriocin producers would be expected to be insensitive to their own bacteriocin (usually referred to as self-immunity). Hence, if a bacteriocin-producing isolate or its CFS inhibits another bacteriocin-producing isolate, which is used as an indicator, it shows that (at least one of) the bacteriocin(s) produced by the former isolate is/are non-identical. On the other hand, the absence of inhibition of the indicator culture by the producer indicates that the secreted inhibitory activity is ineffective, or that the indicator is immune to this inhibition because it is itself producing an identical or almost identical bacteriocin (Hartnett, Vaughan, & Van Sinderen, 2002).

Table 1
Inhibitory activity of the bacteriocinogenic LAB from sourdough raw materials against non-pathogenic food-associated and pathogenic bacteria

Indicator strain[b]	Producer strains[a]															
	WGJ1.2	WGJ5.1	WGJ6.1	WGJ8.1	WGJ17.2	WGJ20.1	WGJ21.2	WGJ28.1	WGJ36.1	WGJ40.2	WGK53	WGW7.2	WGW11.2	WGW33.2	WGWT1.1A	WGX9.1
L. innocua 4202	1.67±0.06[c]	1.47±0.12	1.70±0.10	1.60±0.17	1.87±0.06	2.42±0.08	1.77±0.06	2.33±0.12	1.57±0.12	2.63±0.06	2.53±0.21	1.73±0.06	1.87±0.15	1.57±0.12	1.87±0.06	2.00±0.20
Ent. durans DSM 20633^T	–	–	–	–	–	1.07±0.06	–	1.47±0.12	–	1.37±0.12	1.37±0.06	–	1.37±0.06	1.47±0.12	1.07±0.12	–
Kocuria varians DSM 20033^T	–	–	–	–	–	–	–	–	–	2.23±0.25	1.97±0.15	–	–	–	–	–
Lb. curvatus ssp. *curvatus* ATCC 25601^T	–	–	–	–	–	1.23±0.06	–	1.20±0.00	–	1.40±0.17	1.27±0.12	–	1.40±0.17	1.33±0.15	–	–
Lb. delbrueckii ssp. *bulgaricus* ATCC 11842^T	–	–	–	–	–	1.17±0.06	–	1.40±0.10	–	1.43±0.12	1.47±0.06	–	1.40±0.17	1.27±0.12	–	–
Lb. delbrueckii ssp. *lactis* ATCC 12315^T	–	–	–	–	–	–	–	–	–	–	–	–	–	1.47±0.15	–	–
Lb. fermentum ATCC 14931^T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.07±0.12	–
Lb. helveticus ATCC 15009^T	–	–	–	–	–	0.97±0.06	–	1.13±0.15	–	1.43±0.15	1.37±0.06	–	1.50±0.17	1.07±0.06	–	–
Lb. mindensis DSM 14500^T	–	–	–	–	–	–	–	–	–	0.87±0.12	0.93±0.12	–	1.07±0.12	–	–	–
Lb. paraplantarum DSM 10667^T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.87±0.12	–
Lb. pentosus ATCC 8041^T	–	–	–	–	–	1.27±0.06	–	–	–	1.07±0.12	1.03±0.06	–	0.93±0.12	1.53±0.06	1.27±0.15	
Lb. sakei LMG 2313	–	–	–	–	–	1.77±0.15	1.13±0.06	1.73±0.15	–	2.23±0.15	2.30±0.17	1.77±0.12	1.87±0.06	1.73±0.15	1.6±0.10	1.73±0.15
Leuconostoc mesenteroides DSM 20343^T	–	–	–	–	–	1.70±0.00	–	1.77±0.12	1.53±0.06	1.77±0.15	1.77±0.15	–	2.17±0.12	–	–	–
Pediococcus acidilactici LMG 11384^T	–	–	–	–	–	0.93±0.12	–	1.50±0.00	–	1.17±0.15	1.13±0.15	–	1.53±0.06	1.43±0.15	–	–
L. monocytogenes ATCC 19114^T	2.10±0.10	2.17±0.06	1.93±0.12	2.03±0.12	2.17±0.15	3.07±0.06	2.37±0.06	2.93±0.15	2.17±0.06	3.53±0.23	3.50±0.26	1.87±0.12	2.63±0.06	2.03±0.12	1.37±0.06	1.60±0.17
Ent. faecalis ATCC 29212	–	–	–	–	–	+/–	–	–	–	+/–	+/–	–	+/–	–	–	–
Ent. faecalis MMH 594	–	–	–	–	–	+/–	–	+/–	–	+/–	+/–	–	+/–	+/–	–	–

(–) No inhibition zone.(+/–) Very little inhibition.

[a]Producer strains are termed by the collection reference abbreviation: WGJ17.2, WGJ21.2, WGJ28.1, WGW7.2 and WGW33.2, *Ent. faecium*; WGJ20.1, WGJ40.2, WGK53, WGW11.2 and WGWT1.1A, *Ent. mundtii*; WGJ8.1, *Lb. coryniformis*; WGX9.1, *Lc. garvieae*; WGJ1.2, WGJ5.1, WGJ6.1 and WGJ36.1, *P. pentosaceus*.

[b]The following strains were not inhibited by any BLIS: *Brevibacterium linens* DSM 20158, *Ent. faecium* DSM 20477^T, *Lb. acidophilus* DSM 20079^T, *Lb. alimentarius* LMG 9187^T, *Lb. amylolyticus* DSM 11664^T, *Lb. amylovorus* DSM 20531^T, *Lb. brevis* ATCC 14869^T, *Lb. buckneri* LMG 6852^T, *Lb. casei* LMG 6904^T, *Lb. crispatus* DSM 20584^T, *Lb. delbrueckii* ssp. *delbrueckii* DSM 20074^T, *Lb. farciminis* DSM 20184^T, *Lb. fructivorans* DSM 20203^T, *Lb. hilgardii* DSM 20176^T, *Lb. paracasei* ssp. *paracasei* NCFB 151^T, *Lb. paracasei* ssp. *tolerans* LMG 9191^T, *Lb. paralimentarius* DSM 13238^T, *Lb. plantarum* ssp. *plantarum* ATCC 14917^T, *Lb. reuteri* LMG 9213^T, *Lb. rhamnosus* LMG 6400^T, *Lb. rossiae* DSM 15814^T, *Lb. sanfranciscensis* DSM 20451^T, *Lb. zeae* DSM 20178^T, *Lc. lactis* ssp. *lactis* DSM 20481^T, *Staphylococcus carnosus* ssp. *carnosus* DSM 20501^T, *Weissella confusa* DSM 20196^T, *Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 6558, *Salmonella poona* NCTC 4840, *Escherichia coli* ATCC 8739 and *Clostridium perfringens* NCTC 8237.

[c]Width of the inhibition zone (mm). Results indicate mean±S.D. of three independent experiments.

Adsorption studies

The effect of the pH on adsorption of active proteins onto producer cells was evaluated as reported by Yang, Johnson, and Ray (1992) and Todorov et al. (1999).

Mode of action

To study the effect of the antimicrobial compounds on sensitive cells, 400 µl of the 10-fold concentrated under vacuum (SC110A SpeedVac® Plus), adjusted to pH 6.5 and catalase treated ASs were added to 3.6 ml of BHI and filtered through a 0.22-µm pore size filter (Millipore). The strain used as indicator at this purpose was *Listeria monocytogenes* ATCC 19114^T. The indicator strain was inoculated at a cellular concentration of approximately 10^3 CFU/ml. As a negative control, the supernatant of *Enterococcus casseliflavus* WGW22.1 (a strain which does not produce any antimicrobial compound), similarly treated as above described, was used. Cell suspensions (300 µl each) were transferred into a 100-well plate and cultivated at 30 °C for 72 h using the Bioscreen C MBR (Growth Curves AB Ltd., Piscataway, NJ, USA). The optical density at 600 nm ($O.D._{600}$) was determined at 2-h intervals.

Results

Screening of LAB for antimicrobial compound production

The 137 LAB were initially screened for the antibacterial compound production against two indicator strains (*L. innocua* 4202 and *Lb. sakei* LMG 2313) by means of the agar-spot deferred method. The indicator strains were chosen for their high sensitivity to bacteriocins produced by LAB (Corsetti et al., 2004; Hartnett et al., 2002). In this step the possible inhibitory effect of the organic acids and of hydrogen peroxide was not excluded. Twenty-nine strains tested produced an inhibition zone against one or both indicators. Subsequently, the ASs from the 29 strains were treated with catalase, neutralized, sterilized by filtration and tested by the well diffusion assay against the same two indicators. Sixteen ASs were found to maintain the antimicrobial activity against *L. innocua* 4202 while only 10 (included in the previous 16) were still active against *Lb. sakei* 2313, showing a measurable clear zone around the well (Table 1). The inhibitory activities, as evaluated by the critical dilution assay against *L. innocua* 4202, were as follows: 640 AU/ml for strains WGJ1.2, WGJ5.1, WGJ6.1, WGJ8.1, WGJ17.2, WGJ21.2, WGJ36.1, WGW7.2, WGWT1.1A and WGX9.1; 1280 AU/ml for strain WGW33.2; 2560 AU/ml for strains WGJ20.1, WGJ28.1 and WGW11.2; 5120 AU/ml for strain WGK53; 10,240 AU/ml for strain WGJ40.2.

To better evaluate the inhibitory activity of the 16 producer strains, their ASs were tested against other strains generally found associated with fermented foods and human pathogens (Table 1). All antibacterial substances tested were interestingly active against *L. monocytogenes* ATCC 19114^T while the inhibition of the other indicators was dependent on the producer strain. Enterococci showed the largest spectra of inhibition: *Ent. mundtii* WGJ40.2 and WGK53 inhibited 12 strains; *Ent. mundtii* WGW11.2 inhibited 11 strains; *Ent. mundtii* WGJ20.1 and *Ent. faecium* WGW33.2 inhibited 10 strains; *Ent. faecium* WGJ28.1 inhibited 9 strains; *Ent. mundtii* WGWT1.1A inhibited 7 strains. *Ent. faecium* WGJ21.1 and WGW7.2, *Lc. garvieae* WGX9.1 and *Pediococcus pentosaceus* WGJ36.1 inhibited 3 strains while *Ent. faecium* WGJ17.2, *Lb. coryniformis* WGJ8.1 and *P. pentosaceus* WGJ1.2, WGJ5.1 and WGJ6.1 inhibited only *Listeria* spp. The majority of indicators ($n = 31$) were not inhibited by any producer strain. Among the human pathogens tested, except *L. monocytogenes* ATCC 19114^T, only *Enterococcus faecalis* ATCC 29212 and MMH 594 were slightly inhibited (no clear inhibition was detected) by four and six ASs, respectively.

Effect of enzymes, heat treatment, pH and organic solvent on the antimicrobial activity

The 16 ASs were assayed for sensitivity to hydrolytic enzymes and heat and stability under different pH values and ethanol concentrations. All antibacterial compounds produced by the 16 strains selected were inactivated by proteolytic enzymes (Table 2), indicating that the inhibitory compounds are of proteinaceous nature, a general characteristic of bacteriocins. As protein compounds inhibitory to closely related bacteria can be included in the category of the bacteriocins (Jack, Tagg, & Ray, 1995; Tagg, Dajani, & Wannamaker, 1976) and because the substances of this study have not been characterized for amino acid and nucleotide sequences yet, they will be referred to as bacteriocin-like inhibitory substances (BLIS). All BLIS were insensitive to α-amylase and lipase. Heat treatment progressively reduced BLIS activities till complete inactivation by sterilization (Table 2). All samples retained almost full activity in the pH range (3–11) considered (Table 2). The activity of BLIS WGJ8.1, WGJ17.2, WGJ20.1, WGJ40.2, WGK53 and WGW11.2 was completely retained in the presence of 15% or less ethanol, while the other BLIS activities were partially reduced by ethanol at concentrations of 10% or higher.

Cross-sensitivity test

The results obtained from the cross-sensitivity assay (Table 3) showed that the BLIS produced by 14 of the 16 LAB strains were different from one another. Among pediococci, strains *P. pentosaceus* WGJ1.2 and WGJ5.1 seemed to produce an almost identical active substance, since they showed a similar inhibition spectrum (Tables 1 and 3). Furthermore, BLIS from *Ent. faecium* WGJ21.2,

Table 2
Effect of enzymes, heat treatment, pH, and organic solvent on inhibitory activity of LAB[a]

Indicator strain	Producer strains[b]															
	WGJ1.2	WGJ5.1	WGJ6.1	WGJ8.1	WGJ17.2	WGJ20.1	WGJ21.2	WGJ28.1	WGJ36.1	WGJ40.2	WGK53	WGW7.2	WGW11.2	WGW33.2	WGWT1.1A	WGX9.1
Control (supernatant not treated)	1.67±0.06[c]	1.47±0.12	1.70±0.10	1.60±0.17	1.87±0.06	2.42±0.08	1.77±0.06	2.33±0.12	1.57±0.12	2.63±0.06	2.53±0.21	1.73±0.06	1.87±0.15	1.57±0.12	1.87±0.06	2.00±0.20
Enzymes																
Proteinase K	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Protease B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Trypsin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
α-Amylase	1.50±0.17	1.27±0.06	1.53±0.06	1.40±0.10	1.77±0.06	2.23±0.15	1.60±0.17	2.33±0.12	1.30±0.00	2.43±0.15	2.27±0.12	1.53±0.06	1.83±0.06	1.50±0.17	1.63±0.06	1.77±0.06
Lipase	1.53±0.06	1.13±0.15	1.47±0.12	1.27±0.06	1.77±0.12	2.27±0.12	1.53±0.06	2.33±0.12	1.27±0.06	2.33±0.12	2.27±0.12	1.53±0.06	1.77±0.06	1.30±0.00	1.63±0.23	1.83±0.06
Heat-treatment																
100 °C for 20 min	1.53±0.06	1.17±0.15	1.50±0.00	1.17±0.15	1.40±0.10	2.00±0.20	1.33±0.06	2.23±0.15	1.30±0.00	2.27±0.12	2.27±0.12	1.43±0.15	1.53±0.06	1.27±0.12	1.77±0.06	1.77±0.12
100 °C for 60 min	1.03±0.06	–	1.17±0.15	–	–	1.63±0.06	–	1.87±0.06	–	1.87±0.06	1.87±0.15	–	–	–	1.30±0.00	1.10±0.10
Sterilization	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
pH																
3	1.43±0.25	1.23±0.15	1.40±0.10	1.30±0.00	1.73±0.06	2.27±0.12	1.43±0.12	2.30±0.17	1.27±0.06	2.47±0.12	2.47±0.12	1.23±0.06	1.87±0.06	1.33±0.06	1.60±0.17	1.60±0.17
4	1.47±0.12	1.40±0.17	1.60±0.17	1.43±0.15	1.87±0.06	2.27±0.12	1.43±0.12	2.27±0.12	1.53±0.06	2.43±0.15	2.43±0.15	1.30±0.00	1.77±0.06	1.33±0.06	1.57±0.06	1.63±0.06
5	1.63±0.06	1.43±0.12	1.63±0.23	1.43±0.15	1.87±0.15	2.33±0.12	1.50±0.17	2.23±0.15	1.53±0.06	2.63±0.06	2.47±0.12	1.33±0.06	1.87±0.15	1.47±0.12	1.50±0.17	1.63±0.06
6	1.57±0.06	1.43±0.15	1.63±0.23	1.40±0.17	1.87±0.15	2.43±0.15	1.50±0.17	2.23±0.15	1.57±0.06	2.67±0.12	2.30±0.17	1.30±0.00	1.73±0.06	1.43±0.12	1.53±0.06	1.50±0.10
7	1.60±0.17	1.47±0.12	1.63±0.06	1.43±0.12	1.87±0.06	2.43±0.15	1.87±0.06	2.23±0.15	1.57±0.06	2.63±0.06	2.23±0.15	1.33±0.06	1.97±0.12	1.43±0.12	1.53±0.06	1.53±0.06
8	1.57±0.06	1.47±0.12	1.63±0.23	1.43±0.15	1.77±0.06	2.43±0.15	1.77±0.12	2.27±0.12	1.53±0.06	2.63±0.06	2.30±0.17	1.30±0.00	1.87±0.15	1.40±0.10	1.50±0.10	1.50±0.17
9	1.60±0.17	1.47±0.12	1.63±0.06	1.43±0.15	1.77±0.12	2.33±0.12	1.73±0.06	2.27±0.12	1.53±0.06	2.67±0.12	2.30±0.17	1.30±0.00	1.87±0.15	1.47±0.12	1.53±0.06	1.40±0.10
10	1.60±0.17	1.43±0.12	1.63±0.06	1.43±0.15	1.77±0.12	2.27±0.12	1.43±0.12	2.03±0.06	1.53±0.06	2.43±0.15	2.07±0.12	1.33±0.06	1.87±0.06	1.40±0.10	1.57±0.06	1.30±0.00
11	1.43±0.25	1.23±0.15	1.47±0.12	1.17±0.15	1.73±0.06	2.23±0.15	1.27±0.12	2.07±0.12	1.27±0.06	2.43±0.15	2.00±0.20	1.27±0.12	1.73±0.06	1.33±0.06	1.60±0.17	1.30±0.00
Organic solvent																
C_2H_5OH 5%	1.63±0.06	1.40±0.10	1.43±0.15	1.17±0.15	1.50±0.17	2.03±0.06	1.53±0.06	2.27±0.12	1.23±0.06	2.33±0.12	2.23±0.15	1.53±0.06	1.27±0.06	1.23±0.06	1.77±0.12	1.87±0.06
C_2H_5OH 10%	1.47±0.12	1.13±0.15	1.43±0.15	1.17±0.15	1.53±0.06	2.00±0.20	1.53±0.06	2.03±0.06	1.23±0.06	2.30±0.17	2.23±0.15	1.27±0.12	1.27±0.12	1.20±0.00	1.63±0.06	1.87±0.06
C_2H_5OH 15%	1.47±0.12	1.23±0.06	1.30±0.00	1.17±0.06	1.50±0.17	2.03±0.06	1.33±0.06	2.00±0.20	1.13±0.15	2.30±0.17	2.23±0.15	1.27±0.12	1.23±0.06	1.13±0.15	1.60±0.17	1.57±0.06

(–) No inhibition zone.

[a]All assays were carried out with *L. innocua* 4202 as indicator strain.

[b]Producer strains are termed by the collection reference abbreviation: WGJ17.2, WGJ21.2, WGJ28.1, WGW7.2 and WGW33.2, *Ent. faecium*; WGJ20.1, WGJ40.2, WGK53, WGW11.2 and WGWT1.1A, *Ent. mundtii*; WGJ8.1, *Lb. coryniformis*; WGX9.1, *Lc. garvieae*; WGJ1.2, WGJ5.1, WGJ6.1 and WGJ36.1, *P. pentosaceus*.

[c]Width of the inhibition zone (mm). Results indicate mean±S.D. of three independent experiments.

Table 3
Cross-sensitivity test

Indicator strain	Producer strains															
	WGJ1.2	WGJ5.1	WGJ6.1	WGJ8.1	WGJ17.2	WGJ20.1	WGJ21.2	WGJ28.1	WGJ36.1	WGJ40.2	WGK53	WGW7.2	WGW11.2	WGW33.2	WGWT1.1A	WGX9.1
P. pentosaceus WGJ1.2	–	–	–	–	–	–	–	–	–	–	1.27±0.06[a]	–	–	–	–	–
P. pentosaceus WGJ5.1	–	–	–	–	–	–	–	–	–	–	1.30±0.00	–	–	–	–	–
P. pentosaceus WGJ6.1	–	–	–	–	–	1.41±0.12	–	–	–	–	1.57±0.06	–	–	–	–	–
Lb. coryniformis subsp. *coryniformis* WGJ8.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ent. faecium WGJ17.2	–	–	–	–	–	–	–	–	–	–	1.53±0.06	–	–	–	–	–
Ent. mundtii WGJ20.1	2.11±0.12	1.51±0.12	1.53±0.15	2.03±0.06	1.30±0.00	–	1.92±0.06	–	1.33±0.06	2.07±0.12	–	1.87±0.15	1.43±0.06	–	2.03±0.06	1.60±0.17
Ent. faecium WGJ21.2	–	–	–	2.30±0.17	–	2.15±0.12	–	1.50±0.17	–	2.40±0.17	2.22±0.12	–	–	1.70±0.00	2.03±0.06	–
Ent. faecium WGJ28.1	2.07±0.15	1.80±0.17	1.43±0.15	2.00±0.20	1.51±0.17	1.53±0.06	1.87±0.06	–	1.53±0.06	1.43±0.06	1.57±0.06	–	1.57±0.06	–	2.03±0.06	1.60±0.17
P. pentosaceus WGJ36.1	–	–	–	–	–	–	–	–	–	1.53±0.06	1.63±0.06	–	–	–	–	–
Ent. mundtii WGJ40.2	2.62±0.17	2.21±0.06	1.57±0.06	2.33±0.12	1.63±0.06	1.67±0.15	2.22±0.12	1.45±0.15	1.57±0.06	–	–	2.07±0.06	1.53±0.06	–	2.12.±0.15	1.43±0.06
Ent. mundtii WGK53	2.20±0.00	2.06±0.07	1.87±0.15	2.30±0.17	1.87±0.06	1.87±0.15	2.30±0.12	1.73±0.06	1.87±0.06	2.07±0.12	–	2.00±0.20	1.53±0.06	–	2.22±0.12	2.00±0.20
Ent. faecium WGW7.2	–	–	–	–	–	1.43±0.25	–	1.17±0.15	–	1.27±0.12	1.44±0.12	–	–	–	–	–
Ent. mundtii WGW11.2	–	–	–	1.30±0.00	–	1.33±0.06	–	–	–	1.60±0.17	1.63±0.06	–	–	–	–	–
Ent. faecium WGW33.2	–	–	1.43±0.12	2.00±0.20	–	2.03±0.06	–		1.27±0.06	2.03±0.06	2.03±0.06	–	1.57±0.06	–	2.00±0.20	–
Ent. mundtii WGWT1.1A	–	–	–	2.03±0.06	–	1.87±0.15	–	1.77±0.12	–	2.07±0.12	1.87±0.06	–	–	1.60±0.17	–	–
Lc. garvieae WGX9.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

(–) No inhibition zone.
[a]Width of the inhibition zone (mm). Results indicate mean±S.D. of three independent experiments.

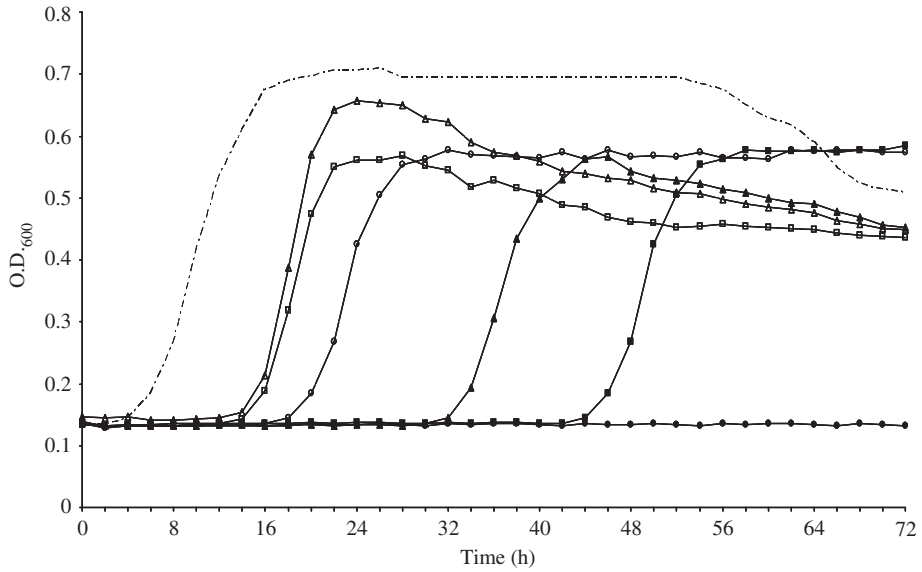

Fig. 1. Inhibition of *L. monocytogenes* ATCC 19114^T growth by addition of concentrated BLIS WGJ36.1 (△), WGJ6.1 (□), WGW33.2 (○), WGJ5.1 (▲), WGJ1.2 (■) and WGJ28.1 (●) to BHI. Control, broken line.

WGJ28.1, WGW7.2 and WGW33.2 and *Ent. mundtii* WGJ20.1, WGJ40.2, WGK53, WGW11.2 and WGWT1.1A were found to be active intra-species.

Mode of action

The mode of action of the ASs was followed for 72 h (Fig. 1) using as indicator *L. monocytogenes* 19114^T which, from the inhibition spectra, resulted to be the most sensitive strain to all 16 BLIS (Table 1). During this period, 11 of the 16 ASs completely inhibited the growth of *L. monocytogenes* 19114^T, thus showing a bactericidal effect (in Fig. 1 only the behaviour of BLIS WGJ28.1 is reported). In the same period, BLIS WGJ1.2, WGJ5.1, WGJ6.1, WGJ36.1 and WGW33.2 are considered to have a bacteriostatic effect on *L. monocytogenes* ATCC 19114^T allowing its growth after 16 (WGJ36.1) or at late 44 (WGJ1.2) h. The CFS from *Ent. casseliflavus* WGW22.1 did not show inhibitory effect since in its presence *L. monocytogenes* ATCC 19114^T showed the same lag phase as in BHI without the addition of concentrated supernatants.

Adsoption of BLIS to the producer cells

Yang et al. (1992) reported that 93–100% of the bacteriocin is adsorbed to the producer cells at pH 6.0 while <5% adsorption took place at pH 1.5–2.0. Under the conditions tested, the activity of ASs of the 16 producer strains obtained after treatment of cells at pH 2.0 was very low, indicating that the inhibitory compound is completely released in the medium during growth and no significant absorption takes place following adjustment of the broth pH to 6.0 before harvesting the cells by centrifugation. Similar findings concerning LAB have been reported by other papers (Corsetti et al., 2004; Todorov, Nyati, Meincken, & Dicks, 2007; Todorov et al., 1999; Todorov, Van Reenen, & Dicks, 2004).

Discussion

In a previous work (Corsetti et al., in press), we found that LAB associated with wheat (*Triticum durum*) grains and non-conventional flours were between less than 1.00 log CFU/g (detected after enrichment procedure) and 2.16 log CFU/g. A total of 356 presumptive LAB isolates were collected and identified. In the present study, the collection of the above LAB isolates, including 137 different strains as shown by RAPD-PCR analysis and geographical origin, was further analysed focusing on their ability to inhibit the bacterial growth by means of bacteriocin production. Sixteen of the 137 strains tested were found to be BLIS-producers. The high percentage (11.68%) confirmed previous observations (Van Sinderen, oral communication) that raw materials harbour higher numbers of bacteriocinogenic strains than fermented foods; e.g. less than 1% of sourdough LAB strains were found to be positive for BLIS production (Corsetti et al., 2004).

The majority of the BLIS-producers of this study (10) were enterococci, 5 strains of *Ent. faecium* and 5 strains of *Ent. mundtii*. Although various bacteriocin-producing enterococci have been collected from plant matrices, *Ent. mundtii* is mostly isolated from plant-related samples, whereas *Ent. faecium* is known to be isolated from a variety of sources, including food products (Klein, 2003). The latter species produces bacteriocins that are expected to be applied for manufacturing meat and dairy products (Franz, Stiles, Schleifer, & Holzapfel, 2003) while bacteriocins from plant-associated *Ent. mundtii* strains might be more suitable for preservation of plant-related foods. In

our work, the strains showing the largest spectra of inhibition and the highest inhibitory activities in terms of AU/ml belonged to *Ent. mundtii* species (WGJ40.2 and WGK53, Table 2). Several bacteriocin-producing strains of *Ent. mundtii* isolated from vegetable matrices have been found, such as strains ST15 and QU 2 from soybean (De Kwaadsteniet, Todorov, Knoetze, & Dicks, 2005; Zendo et al., 2005), strain ATO6 from chicory endive and strain NFRI 7393 from grass silage (Bennik, Vanloo, Brasseur, Gorris, & Smid, 1998). *Lb. coryniformis* is an interesting species from the antimicrobial production point of view, it includes strains able to produce anti-fungal compounds (Magnusson & Schnürer, 2001; Magnusson, Ström, Ross, Sjögren, & Schnürer, 2003) or reuterin, a non-bacteriocin antibacterial substance (Martin et al., 2005). During this study, a strain belonging to the above species was isolated from a durum wheat sample and it was, in fact, a BLIS-producer which, compared to enterococci, showed a weaker antibacterial activity. Several strains of *P. pentosaceus* found to be pediocin-producers are reported in literature (Daeschel & Klaenhammer, 1985; Manca de Nadra, Sandino de Lamelas, & Strasser de Saad, 1998; Piva & Headon, 1994). *P. pentosaceus* is a common starter of meat products and this species includes strains that are able to grow at low temperatures, which would consequently give them a competitive edge against the spoilage organisms and pathogens of refrigerated meats (Yin, Wu, & Jiang, 2003). So far, the only bacteriocin characterized from *Lc. garvieae* is garviecin L1-5 (Villani et al., 2001).

The present results showed that 15 of the 16 BLIS-producers (except *Lc. garvieae* WGX9.1), due to their common presence in food environments and their GRAS status, could be applied for food fermentation. Considering our previous results obtained with *Lactococcus lactis* in sourdough (Settanni, Massitti, Van Sinderen, & Corsetti, 2005), it could be speculated that bacteriocin production may guarantee the persistence of the producer strains and positively influence the interactions among LAB.

None of our BLIS-producer strains was able to clearly inhibit Gram-negative bacteria, at least in the non-concentrated form. The activity of some bacteriocins from LAB against Gram-negative bacteria is an unusual phenomenon, in fact, only a few of them have been reported (Ivanova et al., 1998; Ko & Ahn, 2000; Kuwano et al., 2005; Lee & Paik, 2001; Messi et al., 2001; Todorov & Dicks, 2004b, 2005). Interestingly enough is the strong inhibition of *L. monocytogenes* 19114^T showed by all 16 BLIS-producer LAB. Furthermore, *L. monocytogenes* 19114^T was found to be the most sensitive strain among those tested as indicators, e.g. BLIS WGJ40.2 showed an inhibition of 10,240 AU/ml vs. *L. innocua* 4202, whereas 1,310,720 AU/ml vs. *L. monocytogenes* 19114^T. The ubiquitous nature of *L. monocytogenes*, its hardiness and ability to grow at refrigeration temperatures and anaerobic conditions makes this well recognized pathogen a threat to the safety of public health. Thus, the use of LAB with anti-*Listeria* bacteriocin production as biopreservatives is well accepted (Schillinger, Geisen, & Holzapfel, 1996; Zhu, Du, Cordray, & Ahn, 2005). Many works aimed to the control of *L. monocytogenes* in food matrices by bacteriocinogenic LAB used as protective cultures (Bennik, van Overbeek, Smid, & Gorris, 1999; Castellano, Holzapfel, & Vignolo, 2004), pure bacteriocins added as food ingredients (Laukvá & Czikková, 1999; Schillinger, Becker, Vignolo, & Holzapfel, 2001), immersion in bacteriocin solutions (Bari et al., 2005; Molinos et al., 2005) and active packaging strategies (Ercolini, La Storia, Villani, & Mauriello, 2006; Ming, Weber, Ayres, & Sandine, 1997). The anti-*Listeria* characteristics of our 16 BLIS is of little meaning in cereal-based products such as sourdoughs, but it is of significance in view of their application in the biopreservation of other food ecosystems where *Listeria* spp. may easily grow.

Treatment with α-amylase and lipase did not alter the antibacterial activity of the BLIS, suggesting that the active compounds did not contain a sugar or lipid moiety. Thus, on the basis of their main characteristics such as heat resistance and anti-*Listeria* activity, the 16 BLIS studied can be preliminarily considered as bacteriocins of class II, according to the classification of Nes et al. (1996).

Works are being prepared in order to better characterize the 16 BLIS (optimal conditions for production and amino acid sequence determination) and practical applications of these results are going to be further evaluated in food matrices. Present studies address the use of bacteriocinogenic enterococci as starter cultures in table olive and pediococci and *Lb. coryniformis* WGX9.1 in sourdough fermentation and to the binding of pure bacteriocins to polymeric films for the preservation of smoked salmon during storage. Furthermore, the anti-fungal potential of the 137 LAB strains is being investigated.

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