

Antimicrobial-Producing Lactic Acid Bacteria Isolated from Raw Barley and Sorghum

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ABSTRACT

J. Inst. Brew. 108(2), 169–177, 2002

From a total of four thousand presumed lactic acid bacteria, obtained from raw, unmalted sorghum and barley, 308 isolates were shown to exhibit inhibitory activity against the indicator strain *Listeria innocua* 4202. Six of these inhibitor-producing isolates were selected for further study on the basis of their relatively wide antimicrobial spectrum, which showed that these producers inhibited several Gram-positive bacteria, including a range of beer spoiling bacteria. The proteinaceous nature, anti-microbial activity against closely related species, heat resistance and pH stability of the inhibitory substances produced by these six bacteria identified these compounds as bacteriocins. All six isolates were shown to secrete the inhibitory compounds into the cell free supernatants. Bacteriocins produced by five of the six producers were purified to homogeneity. Further analytic data was obtained for three of the inhibitory compounds by means of mass spectroscopy and/or N-terminal amino acid sequencing.

Key words: Bacteriocin, beer spoilage, lactic acid bacteria, raw barley, sorghum.

INTRODUCTION

Lactic acid bacteria (LAB) are commonly defined as Gram-positive, non-sporulating, catalase-negative, aerotolerant, acid tolerant, nutritionally fastidious, strictly fermentative organisms that lack cytochromes and produce lactic acid as the major end-product of carbohydrate metabolism². They are widely distributed in nature, and are present as natural contaminants on a variety of foods such as milk¹⁷, meat and vegetables⁵⁵, and malted cereals⁴⁵. In such food products LAB have the capacity to perform fermentative activities, which may result in active inhibition of spoilage and pathogenic bacteria. This inhibition is partly due to the production of fermentation end products such as lactic acid, diacetyl, acetaldehyde and acetic acid, which may accumulate to inhibitory levels in certain foods and beverages. In other cases, inhibition may be caused by inadvertent by-products of metabolic activity, e.g. hydrogen peroxide, and the secondary reaction product hypothiocyanate^{55,56}. Antimicrobial activity of LAB may also be produced in the form of small, heat-stable inhibitory

peptides, often referred to as bacteriocins^{14,15,18}. Bacteriocins produced by LAB have been classified into four structural classes, namely Class I, II, III and IV²⁸. Class I and II are small, mainly hydrophobic and heat-stable peptides. The bacteriocins belonging to Class I, also termed lantibiotics (lanthionine-containing peptides with antibiotic activity), represent small peptides that contain di-dehydro-amino acids and thioether amino acids (lanthionine and 3-methylanthionine). Two subgroups have been defined on the basis of their distinctive ring structures: type A comprises screw-shaped, amphipathic molecules with molecular masses of 2,164 to 3,488 Da carrying two to seven net positive charges, whereas type B consists of more globular molecules with molecular masses of 1,959 to 2,041 Da having either no net charge or a net negative charge. Class II is subdivided into three groups; members of Class IIa are represented by the pediocin-like bacteriocins with strong anti-listerial effects that are produced by members of the genera *Enterococcus*⁴³, *Lactobacillus*⁵⁸, *Pediococcus*³⁶ and *Leuconostoc*¹⁹. Class IIb bacteriocins require two peptides for full antimicrobial activity. Finally, Class IIc bacteriocins are secreted by a sec-dependent mechanism (this mechanism is one in which bacteriocin production is shown to be dependent on the expression of a single operon, comprising the structural and putative immunity genes, without the need of dedicated secretion or maturation pathways). Class III are high molecular weight, heat-labile protein bacteriocins, whereas Class IV are complex bacteriocins composed of a protein moiety plus one or more non-proteinaceous additions.

From an industrial perspective bacteriocins are highly valued because of their food-preserving potential³⁹, which has prompted many screening efforts to isolate bacteriocin-producing LAB from a variety of sources (see for example^{6,33,38,44,49}). Bacteriocins can be exploited to inhibit undesirable microorganisms in the fermentation of wine⁴⁰, beer⁴⁷, meat⁵⁷, vegetables¹⁰ and dairy products^{8,52}.

Nisin has found practical application in beer fermentation and wine making. Various studies have indicated that nisin can inhibit beer spoilage organisms without exhibiting detrimental effects on the brewing yeasts^{47,48}. Moreover, nisin addition during the fermentation process appears to have no adverse effect on the taste of the beer⁴⁶.

In previous studies we have identified and analysed bacteriocin-producing LAB from malted barley samples^{44,59}. These studies have shown that bacteriocins are produced by various LAB species such as *Lactobacillus sakei*, *Leuconostoc mesenteroides* and *Enterococcus faecalis*. The objective of this study was to analyse raw, unprocessed cereals as a possible source for bacteriocin-

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producing LAB and to characterise the identified bacteriocins with respect to their inhibitory spectrum and physical properties.

MATERIALS AND METHODS

Bacterial cultures and media

All bacterial cultures used (Table I) were maintained as frozen stocks in 100% glycerol at -20°C and were propagated twice in broth for 16 h before experimental use. LAB isolates were selected and cultivated on de Man Rogosa Sharpe Agar (MRSA, Oxoid) at 30°C . Cultures, seen to be inhibitory when cultivated on MRSA, were transferred to TSA/YE (tryptone Soya Agar (Difco) supplemented with 0.6% (w/v) yeast extract) plates supplemented with beta-glycerophosphate as a buffering agent. M17 broth (Oxoid) supplemented with 0.5% glucose (GM17) was used to cultivate lactococcal strains. The indicator strain *Listeria innocua* 4202 was propagated at 30°C in TSA/YE (Difco). Isolates M36 and S33 were propagated at 30°C in tryptone yeast extract (TY) broth (2.5 g L^{-1} of tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} of glucose, 19 g L^{-1} beta-glycerophosphate, 0.25 g L^{-1} of MgSO_4 , 0.05 g L^{-1} of MnSO_4 ; pH 6.75). Unhopped wort used for the cultivation of the six selected bacteriocin-producing LAB strains was obtained from the University College Cork pilot plant microbrewery (specific gravity of the wort was 1.061 (15 °P)) and was made using a Congress mashing procedure (Analytica EBC 4.5.1). Solid media were prepared by the addition of 1.5% agar to the appropriate broth and overlay media by the addition of 0.7% agar. Anaerobic conditions were established using Anaerocult A gas packs (Merck). Bacterial cultures, other than LAB, were obtained from the University College Cork culture collection and were propagated and grown with agitation in TSB/YE (tryptone Soya Broth (Difco) supplemented with 0.6% (w/v) yeast extract) at 37°C . *Micrococcus luteus*, *Pseudomonas fluorescens* and *Pseudomonas putida* were propagated at 30°C . *Clostridium* species were cultured in reinforced clostridia media (LAB M).

Selection procedure for LAB from raw cereals

Red sorghum and white sorghum samples were sourced from grain stores in South Africa, while wheat and barley samples originated from Iran (as the microflora of grain sourced locally had previously been examined in detail^{42,45}, it was decided to screen cereal grain from more exotic sources in an attempt to isolate different LAB). LAB cultures were isolated from wheat, barley and sor-

ghum by adding 5 g of sample aseptically to 50 mL sterile quarter strength Ringer's solution followed by overnight incubation at 30°C with constant agitation. Serial decimal dilutions of samples were then prepared in Ringer's solution (Merck), and appropriate dilutions were plated on MRS agar plates containing cycloheximide ($100\text{ }\mu\text{g mL}^{-1}$) to prevent fungal growth, and to select for LAB.

Detection of bacteriocin activity

Isolated colonies of the assumed LAB isolates were screened for antimicrobial-producing activity essentially using the deferred antagonism assay³⁰. Colonies initially seen to inhibit the indicator culture (*L. innocua* 4202, which was obtained from the UCC culture collection) when growing on MRSA were transferred to buffered MRSA plates. These buffered plates allowed detection of isolates, which were inhibitory due to their production of acid (rather than a bacteriocin-like inhibitory substance). This inhibition was scored on an abstract scale as follows: -, No inhibition; +, zone of inhibition 1 to 5 mm; ++, zone of inhibition 5 to 10 mm; +++, zone of inhibition > 10 mm. Potential inhibitor-producing cultures were purified from master plates and used for further characterisation. Sensitivity of the inhibitory activity to proteolytic enzymes or catalase was determined as described previously⁴⁴. The enzyme (Sigma Chemical Company) solutions used in these assays were as follows: catalase and proteinase K in distilled water, pepsin in 20 mM sodium phosphate buffer, pH 7. The final enzyme concentration used in all assays was 10 mg mL^{-1} .

Strain characterisation

Carbohydrate fermentation profiles and biochemical characteristics were determined using API 50 CHL for lactobacilli, pediococci and leuconostocs and API 20 Strep for streptococci and enterococci (Biomérieux, France). The production of gas from glucose was examined by a previously reported method⁴⁴. Genomic DNA extraction, polymerase chain reactions and sequencing of the 16S rRNA-encoding DNA was performed as described by O'Mahony⁴⁴. Data base searches were carried out using the latest release of non-redundant DNA sequence database present at the NCBI website located at <http://www.ncbi.nlm.nih.gov/BLAST>¹.

Characterisation of antimicrobial agents

Aliquots (1.5 mL) of exponentially growing (O.D ~ 1.4) LAB cultures were centrifuged at 10 000g for 1 min followed by filter sterilisation of 300 μl of the resulting cell-free supernatant (CFS) through 0.45 μm Spin X Cen-

TABLE I. Characterisation of presumptive bacteriocin-producing LAB.

Strain	Source	Shape	Growth range (°C)	Optimum temperature	Gas from glucose	Identity from API profiles	Identity from 16S rRNA	Base pairs sequenced and % homology
A33	Barley	Coccus	4 – 48	37	No	<i>E. faecalis</i>	<i>E. faecium</i>	448, 99%
T33	Sorghum	Coccus	4 – 48	37	No	<i>E. faecium</i>	<i>E. faecalis</i>	383, 99%
4	Sorghum	Coccus	4 – 48	37	No	<i>E. faecalis</i>	<i>E. mundtii</i>	446, 99%
S33	Barley	Coccus	10 – 42	30	No	<i>L. lactis</i> subsp. <i>cremoris</i>	<i>L. lactis</i>	379, 100%
M30	Barley	Coccus	10 – 42	30	No	<i>L. lactis</i>	<i>L. lactis</i>	307, 100%
M36	Barley	Coccus	10 – 42	30	No	<i>L. lactis</i>	<i>L. lactis</i>	398, 100%

trifuge Tube Filters (Corning Costar) at 8 000g for 15 sec. Bacteriocin activity in the CFS was quantified by an adaptation of the critical dilution method for quantifying of bacteriocin activity as described by Joerger and Klaenhammer²⁶ using *L. innocua* 4202 as the indicator culture. The relative bacteriocin concentration was defined as the reciprocal of the highest dilution showing complete inhibition of the indicator lawn and was expressed in activity units (AU) per mL.

Temperature sensitivity was tested by exposing aliquots of filter sterilised bacteriocin-containing CFS, freshly obtained from a particular LAB isolate, to various temperatures for varying times. Following the temperature treatment, the bacteriocin activity of the treated samples was quantified as outlined above and compared to the activity of the untreated samples. In order to determine the effect of pH on inhibitory activity, filter sterilised CFS of a particular isolate was pH-adjusted to the desired pH (ranging from pH 2 to pH 11) using 5M HCl or 5M NaOH, and subsequently incubated for one hour at 30°C and assayed for bacteriocin activity as described above.

Production studies and inhibitory spectrum of the bacteriocin-producing LAB

The growth rate of each of the LAB inhibitor-producers was measured turbidometrically (OD₆₀₀) for 18 h at 30°C for the lactococcal strains and at 37°C for the enterococcal strains. Aliquots (1.5 mL) were collected from broth at several time points and the CFS was used for a quantitative assay of bacteriocin activity as outlined above. The activity spectrum of each of the inhibitor-producing LAB strains was tested at different time points and arbitrarily quantified (so that the timing of maximum bacteriocin production for each strain could be established) for both cell spots and their CFS as described above against various Gram-negative and Gram-positive bacteria, including beer spoilage bacteria.

Bacteriocin purification and characterisation of the purified peptide

Enterococcal strains. Bacteriocin activity of the enterococcal strains A33, 4 and T33 was purified from the CFS of early stationary phase cultures of the relevant producing strains essentially as described previously^{14,58}. Briefly, the inhibitory activities were precipitated from the CFS following the addition of ammonium sulphate (40% w/v saturation). The precipitated proteins were collected by centrifugation (10 000g for 30 min), resuspended in and dialysed against 20 mM sodium phosphate buffer (pH 6), and purified using three consecutive chromatography steps: a cation exchange (SP Sepharose®, Pharmacia), a hydrophobic interaction (Octyl Sepharose® CL-4B, Pharmacia) and finally reversed phase chromatography (see below).

Lactococcal strains. Prior to inoculation, the growth medium for the lactococcal strains S33 and M36 was cleared of contaminating proteins by passing it through 500 g of XAD-16 beads (Sigma-Aldrich Co. Ltd., Dorset, England) to which hydrophobic proteins bind. Following overnight growth of the bacteriocin-producing strains, the culture was centrifuged (10 000g for 15 min) to remove cells. The supernatant was then passed through 500 g of XAD-16 beads at a flow rate of approximately 15 mL

min⁻¹, allowing the bacteriocin to bind. The column was subsequently washed with 40% ethanol, followed by elution of the inhibitory activity using 70% v/v 2-propanol in 10 mM acetic acid (pH 2). The 2-propanol was removed by rotary evaporation using a Buchi apparatus (Buchi AG, Flawil, Switzerland), and the resulting bacteriocin-containing solution was dialysed against 2 mM sodium phosphate buffer (pH 7) prior to reversed phase chromatography (see below).

Reversed phase chromatography. For both purification methods described above the partially purified samples were freeze-dried and the resulting powder was resuspended in 150 µl of 6 M urea before being subjected to reversed phase chromatography. Complete purity was achieved by several (usually three) consecutive runs of the reversed phase chromatography step using a Beckmann Ultrasphere ODS (5 µm, 4.6 mm × 25 mm) C₁₈ column on a HPLC system (Beckmann). The first run was seen to remove impurities which appeared as shoulders on the peak, the second run removed more of the non-active proteins while the third (and usually final) run was seen to yield a very sharp narrow protein peak. The bacteriocin activities were eluted with an increasing linear gradient (10 to 80% (v/v) in case of isolates A33, 4, T33, or 10 to 100% (v/v) in case of isolates S33 and M36) of acetonitrile in an aqueous trifluoroacetic acid (0.1%) solution. Absorption was monitored at 280 nm to detect protein peaks, and fractions of 1.0 mL were collected. Protein concentrations were determined using the BIO-RAD protein assay kit (Biorad Laboratories Ltd.). All fractions were assayed for bacteriocin activity against the indicator culture *L. innocua* 4202 as described above. N-terminal amino acid sequence was performed by automated Edman degradation with a model 473A sequencer (Applied Biosystems, California, USA). Mass determinations were performed using a FiniganMat (Laser Mat™) mass spectrometer and a MALDI-TOF (Voyager-DE™ RP, Perseptive Biosystems).

RESULTS

Detection of antimicrobial producing LAB

Putative antimicrobial-producing LAB isolated from raw/unprocessed cereals were detected using the deferred antagonism assay on the basis of their ability to inhibit growth of the indicator strain *L. innocua* 4202. In total 308 inhibitor-producing bacteria, which were presumed to be LAB, were isolated from four thousand (a thousand randomly picked isolates from each of the four cereals mentioned below) screened isolates. Of these 308 inhibitor-producing isolates, 135 were obtained from barley, 63 from wheat, 52 from red sorghum and 58 from white sorghum.

Sensitivity of antimicrobial compounds to proteases and catalases

Catalase and protease tests showed that the vast majority of the 308 inhibitor-producing LAB inhibit *L. innocua* 4202 by means of hydrogen peroxide rather than a proteinaceous substance (results not shown). In fact, it appeared that only 112 of the inhibitor-producing LAB (of

an overall total of four thousand isolates) produced a bacteriocin-like substance(s), and from these 112 isolates, only six (named A33, T33, 4, M36, M30, and 4) were taken for further characterisation. These six bacteriocin-producing isolates were chosen as they (1) consistently released their inhibitory activity into the CFS (see below), and (2) were exhibiting a homofermentative metabolism (homofermentative metabolism is important as its primary product is lactic acid as opposed to heterofermentative metabolism which produces CO₂, acetic acid, ethanol,

mannitol and lactic acid, many of which are undesirable in the final product).

Characterisation of presumptive bacteriocin producers

The six presumptive bacteriocin producers were characterised and identified to species level utilising carbohydrate fermentation profiles, biochemical characteristics and 16S rRNA sequencing. Table I details the characteristics of these six producers, which are representatives of

TABLE II. Effect of temperature treatments on inhibitory activity of CFS obtained from various LAB isolates using *L. innocua* 4202 as the indicator organism.

Temperature treatment	Inhibitory activity (AU/mL)					
	A33	T33	4	S33	M30	M36
Control	25600	12800	25600	25600	25600	25600
50°C × 60 min	25600	12800	25600	25600	12800	25600
65°C × 60 min	25600	12800	12800	25600	12800	25600
80°C × 60 min	25600	12800	12800	12800	12800	25600
90°C × 60 min	3200	6400	6400	12800	12800	12800
100°C × 60 min	1600	6400	6400	12800	6400	12800
115°C × 10 min	800	1600	6400	6400	6400	12800
121°C × 15 min	800	800	1600	6400	6400	12800
-20°C × 31 days	25600	12800	25600	25600	25600	25600
4°C × 31 days	3200	6400	6400	3200	1600	3200
13°C × 31 days	200	400	400	200	0	200
30°C × 31 days	1600	800	800	400	400	400
37°C × 31 days	1600	400	800	400	400	400
42°C × 31 days	1600	0	400	100	200	400

TABLE III. The effect of pH on the inhibitory activity of CFS obtained from LAB isolates tested against *L. innocua* 4202 as the indicator.

pH	Inhibitory activity (AU/mL)					
	A33	T33	4	S33	M30	M36
2	800	800	1600	1600	800	1600
3	1600	800	1600	3200	1600	3200
4	3200	1600	3200	3200	3200	3200
5	3200	3200	3200	3200	6400	3200
6	12800	12800	3200	6400	6400	6400
7	12800	12800	3200	1600	3200	1600
8	12800	12800	3200	1600	1600	1600
9	6400	6400	1600	800	400	800
10	800	1600	800	400	400	400
11	200	0	0	0	0	0

TABLE IV. Inhibitory spectrum of CFS from bacteriocin producing LAB isolates.

Indicators	Inhibitory activity of producing strains ¹					
	A33	T33	4	S33	M36	M30
<i>L. innocua</i>	+++	+++	+++	+++	+++	+++
<i>Lb. sakei</i> ²	++	++	++	++	++	++
<i>B. coagulans</i>	++	++	++	++	++	++
<i>B. cereus</i>	-	-	-	-	+	-
<i>B. subtilis</i>	-	+	-	-	+	-
<i>S. aureus</i>	-	-	-	-	-	-
<i>M. luteus</i>	-	-	-	-	-	-
<i>L. lactis</i> MG 1363	-	+	-	-	+	+
<i>E. coli</i>	-	-	-	-	-	-
<i>E. coli</i> 555	-	-	-	-	-	-
<i>S. mutans</i>	-	-	-	-	-	-
LT2	-	-	-	-	-	-
<i>K. pneumoniae</i>	-	-	-	-	-	-
<i>C. freundii</i>	-	-	-	-	-	-
<i>C. putita</i>	-	-	-	-	-	-

¹ + → +++ Indicates increasing zone of inhibition; - indicates no inhibition.

² *Lb. sakei* : *Lactobacillus sakei*.

TABLE V. Inhibitory spectrum of cereal LAB against beer spoilage LAB.

Indicator strain	Species	Source	Inhibitory activity of producer strains ¹					
			A33	T33	4	S33	M36	M30
101	Unknown	Beer	-	++	++	+++	+++	+++
102	<i>Lb. brevis</i>	Beer	-	+++	+	++	++	+
103	<i>Lb. brevis</i>	Beer	+++	++	++	+++	+++	+
104	<i>Lb. brevis</i>	Beer	++	++	+	++	++	++
105	<i>Lb. lactis</i>	Beer	++	+++	++	+	++	+
106	<i>Lb. cellobiosus</i>	Beer	-	+++	++	+++	+++	-
107	<i>Lb. paracasei</i>	Beer	+	++	+	+	++	+
108	<i>Lb. brevis</i>	Beer	++	++	+	++	+	++
109	<i>E. faecalis</i>	Beer	+	+	+	++	++	+
110	<i>L. collinoides</i>	Cider	++	++	+	+	+	-
111	<i>Lb. brevis</i>	Beer	-	++	+	++	++	++
112	<i>Lb. brevis</i>	Cider	+++	++	+	++	++	-
113	<i>P. dammosus</i>	Stout	-	-	-	-	+	-
114	<i>P. dammosus</i>	Stout	+	-	-	-	+	-
115	<i>Lb. plantarum</i>	Beer	++	++	+	++	++	++
116	<i>Lb. malefermentens</i>	Beer	+++	++	++	++	++	+
117	<i>Lb. brevis</i>	Fermenter sample	++	+++	+	++	+++	++
118	<i>Lb. sakei</i>	Beer	+++	++	++	++	++	++
119	Unknown	Beer	++	++	+	++	+	++
120	Unknown	Beer	+	++	+	+	+	++

¹ + → +++ Indicates increasing zone of inhibition; - indicates no inhibition.

two homofermentative genera of LAB, i.e. *Lactococcus* (in the case of isolates S33, M30 and M36) and *Enterococcus* (in the case of isolates A33, T33 and 4). The six LAB isolates were shown to have a relatively wide growth temperature range. All enterococci were capable of growth between 4°C and 48°C, while the lactococci displayed growth at temperatures ranging from 10°C to 42°C.

Characterisation of the inhibitory activity

The CFS of growth medium from each of the six presumptive bacteriocin-producers was shown to inhibit growth of *L. innocua* 4202, which demonstrated that these isolates release (some of) their inhibiting activity in the growth medium. Enzyme assays confirmed that the observed inhibition of the indicator strain was protease sensitive and therefore assumed to be due to a bacteriocin-like substance.

The stability of the secreted inhibitory compounds was tested using different temperature treatments (Table II). The inhibitory activity was shown to be relatively unaffected following heat treatments at temperatures between 50°C and 80°C. The inhibitory compounds produced by isolates M36 and S33 were seen to be the most stable to heat treatments up to and beyond 80°C. M36 maintains its activity at 12,800 AU/mL even after treatment at 121°C for 15 min, a property which is typical for bacteriocins^{26,44}.

The observed protease sensitivity and stability at high temperatures therefore conclusively identifies these compounds as bacteriocins.

Partial or even complete loss of activity was seen to occur for all bacteriocins following exposure to moderate temperatures for prolonged periods of time. This may be caused by the presence of very small amounts of protease and/or peptidase activity in the CSF, most likely produced during growth (and limited lysis) of the LAB isolate in this medium. The stability of the inhibitory activity was tested at different pH values (Table III). The activities obtained in Table III were much lower than those in Table II

the reason for this could be that all the isolates were maintained at 30°C for 60 min when being assayed. If any residual protease was present in the CFS when assaying the effect that the different pH levels have on activity, it would be most active at 30°C. This would account for the large differences in activities between Tables II and III. The bacteriocins produced by isolates A33, T33, and 4 showed greater pH tolerance and stability than those secreted by isolates M30, M36, and S33.

Production studies

Antimicrobial activity produced in the CFS by each of the six producers was detectable after 4 to 5 h (early log phase: OD_{600nm} was between 0.5 and 0.6) following incubation at 30°C (lactococcal isolates) or 37°C (enterococcal isolates) with maximum activity reached between 14 and 18 h of growth (late log phase: OD_{600nm} was between 2.5 and 3.0). The inhibitory activity produced by isolates M30, M36 and S33 was seen to decrease by 50% after 20 h (stationary phase) whereas isolates A33, T33 and 4 retained maximum activity throughout the stationary phase.

TABLE VI. Production of bacteriocin in wort over a two week period.

Day	Inhibitory activity of producer strains ¹					
	A33	T33	4	S33	M36	M30
1	+	-	-	+	-	-
2	+	-	-	+	-	-
3	+	-	-	+	-	-
4	++	-	+	+	-	-
5	++	+	+	+	-	-
6	++	++	+	+	-	-
7	++	++	+	++	-	-
8	++	++	+	++	-	-
9	++	++	+	++	-	-
10	++	++	-	++	-	-
11	++	++	-	++	-	-
12	++	++	-	++	-	-
13	++	++	-	++	-	-
14	++	++	-	++	-	-

¹ + → ++ Indicates increasing zone of inhibition; - no inhibition.

Table VII. Comparison of amino acid sequence obtained for A33 with mundticin.

Bacteriocin ¹	Sequence
A33	KYYNGVSCNKKGCSVDWGKAIGHIINN
Mundticin ⁴	KYYNGVSCNKKGCSVDWGKAIGHIINNNSAANLATGGAAGWSK
T33	ATRSYNGVYCNNSKCVWNWGEAKE
Enterocin P ⁹	ATRSYNGVYCNNSKCVWNWGEAKENIAGIVISGWASGLAGMGH
Enterocin A ³	TTHSGKYYNGVYCTKNKCTVDWAKATTTCIAGMSIGGFLGGAIPGKC

¹ Aymerich et al., (1996)³, Cintas et al., (1997)⁹, Bennik et al. (1998)⁴.

Cross sensitivity

The cross sensitivity of the barley and sorghum-derived bacteriocin producers was examined. The producers isolated in this study were challenged by known bacteriocin-producing strains isolated in a previous study^{44,59} and *vice versa* 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. Oppositely, 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. The results obtained showed that the bacteriocins produced by the six isolates were different from one another and from those identified in a previous study. This earlier study on malted barley as a possible source of bacteriocins yielded thirty-three inhibitor producing LAB, eleven of which were shown to be bacteriocin-producers^{44,59}.

Inhibitory spectrum

The sensitivity of various Gram-positive and Gram-negative bacteria to the CFS of the six producing isolates was determined using the deferred antagonism assay. Table IV represents the results obtained using a variety of bacteria that are not normally associated with beer spoilage. The inhibitory spectrum of the CFS obtained from the six isolated bacteriocin-producing LAB tested against these bacteria included most notably *B. cereus* and *B. subtilis*, which were consistently inhibited by isolates T33 and M36, although not to the same extent as some of the other bacteria tested. None of the Gram-negative bacteria tested were inhibited by CFS obtained from any of the bacteriocin producers. This result in itself is not surprising as bacteriocins produced by LAB are not known to inhibit Gram-negative species.

Table V shows the activity of the CFS of the producer strains when tested against known beer spoilers. CFS from bacteriocin-producer M36 was shown to have the broadest inhibitory spectrum of the producer strains against these bacteria, while the CFS of producers, S33, 4, T33, A33 and M30 exhibited a narrower spectrum.

Production in wort

In order to determine if the LAB isolates also produce their bacteriocins in unhopped wort, they were grown in this medium, which upon removal of the cells was then tested for bacteriocin activity. Two strains, S33 and A33,

were shown to consistently produce their inhibitory activity in wort over an extended period of time. On the other hand, isolates 4 and T33 required several days of growth before bacteriocin production became obvious, although in the case of isolate 4 this property was again lost after day 10. The two *L. lactis* strains M30 and M36 did not produce their inhibitory compounds in wort under these conditions (Table VI). However, the addition of 50 µl of CFS from an overnight culture of bacteriocin-producing M30 or M36 to their corresponding non-producing strain in wort was shown to induce bacteriocin production of M30 or M36, respectively, in this medium and this bacteriocin-positive phenotype was seen to be stable following subsequent incubations. However, this procedure did not allow bacteriocin production of isolate 4 in wort. The observed production behaviour of isolates M30 and M36 is typical of many bacteriocin-producers, which regulate bacteriocin production by means of inducing peptides^{11,12,13,41}.

Purification and characterisation of the bacteriocins

In order to purify the inhibitory activity from the CFS of each of the six producers, two purification protocols were attempted (see methods). Both methods employed reversed phase HPLC chromatography as the final purification step. This resulted in a single active fraction for isolates A33 and T33. In the case of isolate M36 it was found that inhibitory activity appeared to be lost during the final purification step. However, inhibitory activity could be retrieved by pooling two different HPLC fractions, designated here as T40 and T60. This is typical for Class IIB bacteriocins, which require two different peptides for bacteriocin activity. No fractions with activity were obtained for isolates S33 or 4 after the HPLC step so further characterisation was not possible. There was no attempted purification of M30 for two reasons (i) it had a weaker inhibitory spectrum than M36 and (ii) it was also a *L. lactis* strain. Successful N-terminal amino acid sequences were obtained for isolates A33 (*Ent. faecium*) and T33 (*Ent. faecalis*). A comparison between the N-terminal amino acid sequence and mass spectrometry value of the bacteriocin A33 identified in this study and that of an apparently identical, previously isolated bacteriocin is shown in Table VII. Mass determinations were obtained for A33,

TABLE VIII. Molecular weight(s) of bacteriocins purified from HPLC.

Bacteriocin	Molecular weight (Da)
A33	4374.0
T33	4425.0
M36 (T40)	No molecular weight obtained.
M36 (T60)	2865.8

T33 and one component of the two-component bacteriocin from the producer *L. lactis* M36 (Table VIII).

DISCUSSION

In the brewing industry grain quality is an important parameter in production of a quality end product. This quality can at various stages (from the field to the malting stages) become diminished due to the contamination of the cereal with undesirable microorganisms, often resulting in an increased turn-over time and product inconsistency affecting both the malting and brewing process.

A range of microorganisms can affect malt and beer quality: bacteria, yeasts and moulds are commonly found to contaminate the cereal(s) used in brewing. Heterofermentative LAB belonging to the genera *Lactobacillus* and *Pediococcus* are the major spoilage organisms throughout the brewing process, either before, during or following fermentation, and may result in significant economic losses to a brewery²⁴. Beer spoil by *Lactobacillus* species during the fermentation process is characterised by excess acidity and turbidity, while carrying off-flavours, such as those caused by diacetyl. It will also contain higher levels of 2,3-butanediol²².

Contaminations with *Pediococcus* species occur mostly in breweries practising bottom-fermentation, and are rare in top-fermentation systems. Symptoms of pediococcal contaminations, the so-called 'sarcina-sickness', are that such afflicted beers become turbid, with granular sediment, while exhibiting excess acidity and increased levels of diacetyl. Contamination levels of 2×10^4 pediococcal cells mL⁻¹ may cause diacetyl levels to exceed 0.36 ppm, which is many times higher than the taste threshold (taste threshold for diacetyl is about 0.03-0.05 ppm) for this compound⁴. Some *Pediococcus* strains also produce 'rope' (a complex polysaccharide slime) and haze in the final product²².

The analysis described in this paper was intended to isolate bacteria from raw cereals, which possess the ability to control bacterial beer contaminants and beer spoilage microorganisms. Research has shown that bacteria are numerically the most dominant microflora during the early stages of kernel development^{16,21,29}, while at the final stage of malting (after kilning) the surviving bacteria were shown to be the hardier remnants of the original microbial field flora^{31,32,50}.

LAB strains with antimicrobial activity, but isolated from a malted barley source, have been described and characterised previously^{44,59}. It was for this reason that raw cereals as opposed to malted cereals were selected as a possible source for novel antimicrobial-producing LAB so that this "missing flora" between the field stage and the malting stage could be explored. Bacteriocin-producing LAB isolated from raw cereals would also be expected to be suitable "natural" inoculants for the inhibition of undesirable bacteria at the malting stage, as spoilage effects start with the raw material. These bacteria may have developed adaptive mechanisms to compete efficiently with cereal-associated spoilage bacteria and would therefore be the preferred "starter brewing LAB" as opposed to non-cereal isolated LAB. These LAB may confer a selective advantage over other LAB found in the brewing process

as they have been shown to produce bacteriocin(s) at an early stage of the brewing process. The advantage of using these LAB is that they are 'killed off' when the wort is boiled and the bacteriocins (which have been shown to be heat stable) they produce would remain and could prevent later contamination of the wort and/or beer.

This study describes the isolation and characterisation of six bacteriocin producers from wheat, barley and sorghum, representing single isolates of *Ent. faecalis*, *Ent. faecium*, *Ent. mundtii* and three *L. lactis* isolates, all of which were different from those isolated previously from malted cereals. This would indicate that a wide variety of bacteriocin-producing LAB are present on raw or malted cereals, which therefore represent an abundant resource of such potentially useful bacteria. However, the percentage of LAB that was found to produce bacteriocins is rather low compared to other sources⁶⁰. In addition, it was shown that the sequenced bacteriocins of A33 and T33 represented bacteriocins, which had been identified in isolates of the same species from different sources^{4,5}. In a previous study by Vaughan⁵⁹, eleven bacteriocins were characterised to the level of N-terminal sequence. Of these eleven bacteriocins only two (sakacin 5T and sakacin 5X) were found to be novel. Therefore, it appears that the number of different bacteriocins is rather limited or that the screening procedures used will only allow the identification of a certain selection of bacteriocins.

M36 however was shown to produce a two component bacteriocin, with one component having a molecular weight of 2865.8 Da. Only a few *L. lactis* are known to produce two-component bacteriocins⁶¹. Lactococcin G⁴² was the first reported bacteriocin that requires the complementary action of two distinct peptides. The two peptides termed α_1 and β have molecular weights of 4,376 Da and 4,109 Da. The large difference in molecular weight between Lactococcin G and that isolated from M36 suggests that the bacteriocin produced by M36 is therefore different. The T60 peptide component of M36 was seen to have a similar molecular weight to one of the two peptides (Ltn A1 3322.34 Da, Ltn A2 2847.47 Da) of the two-component bacteriocin lacticin 3147⁵³, but from cross sensitivity tests it has been shown that the bacteriocin produced by these two strains is different. Also the Ltn A1 component of lacticin 3147 is seen to have some residual inhibitory activity on its own, this is not seen to occur with the T40 component of M36. The inhibitory compounds produced by the six isolates demonstrated a high resilience to heat treatments ranging in temperature from -20°C to 121°C (Table II). The *Ent. faecium* isolate A33 showed the greatest stability of the enterococcal strains with *L. lactis* M36 proving to be the most stable of the lactococcal strains. The bacteriocins were shown to be stable over a broad pH range with all peptides maintaining some antimicrobial activity within the pH range of pH 2 to pH 10. Following incubation at extreme pH values (pH 2 - 3 and pH 9 - 11) a significant reduction in bacteriocin activity was observed, similar to results reported previously^{20,25,62}, with only *Ent. faecium* A33 seen to retain residual activity at pH 11. The observed pH optimum of the bacteriocin activity obtained from the enterococcal strains is similar to that reported for some enterocins²⁵, but higher than that recorded for other enterocins such as enterocin 1146²⁰, which

is also stable at acid pH. The loss in activity by the lactococcal strains at lower pH values is not wholly in agreement with that of other results, as other lacticins, such as lacticin BH5²³ are known to maintain stability over a wide acidic pH spectrum (pH 2 – pH 5).

The inhibitory spectrum of the CFS, obtained from each of the six isolated bacteriocin-producing bacteria, against a wide variety of known beer spoilage microorganisms as well as other bacteria showed that these compounds acted very efficiently to inhibit growth of these Gram-positive bacteria.

The ability of four of the six producers to produce bacteriocins in wort is of great interest and importance with regards to the possible application of these strains in the brewing environment. Interestingly, it was seen that two of the strains M30 and M36 did initially not produce in wort. However, this bacteriocin-negative phenotype was reversed upon the addition of small amounts of CFS from a bacteriocin-producing variant of the same strain. This phenomenon may on one hand hinder their possible application in the brewing environment, but may on the other hand allow the inhibitory activity of the strain to be precisely controlled by addition of the inducing compound. Thus, it appears feasible to use controlled bacteriocin-producing LAB as biological acidifiers of wort, whose bio-preserving effect will last into and beyond the fermentation stage even though the producing bacteria may not have survived.

From mass spectrometry and amino acid sequences it was shown that A33 produced a bacteriocin that was identical to the bacteriocin mundticin. Molecular weight of T33 and M36-T60 are in keeping with those of known enterocin(s)⁹ and two component lacticins⁵¹, respectively. It was not possible using the conventional methods used for the other bacteriocins in this study to obtain an N-terminal sequence for M36, as M36 probably belongs to the class of antimicrobials known as lantibiotics. This group of post-translationally modified peptides, which includes nisin, is characterised by the presence of a high proportion of unusual amino acids, including lanthionine and β -methylanthionine. If M36 therefore contains these unusual amino acids, they are more than likely the reason why no N-terminal sequences were obtained for M36 (T40 and T60). Several bacteriocins from the genus of *Enterococcus* have been described^{27,30,37,39,54}, yet due to their association with microbial spoilage and implication in outbreaks of food-borne illness, their possible use as bacterial adjuncts in the brewing industry would be questionable. Lactococcal species on the other hand are well-known and widely used starter cultures in numerous fermentation procedures⁷ and would not suffer from such a drawback.

In conclusion, the bacteriocin-producing LAB isolated and characterised during this study have demonstrated themselves to be active inhibitors of common food and beer spoilage bacteria that may compromise beer quality and safety. The practical application of such strains in the brewing process will have to be investigated further by means of small-scale brews before being tested on a larger scale. The development of a bacteriocin-producing malting and/or brewing inoculant is therefore still some time away but remains promising.

ACKNOWLEDGEMENTS

This work was supported by the Department of Agriculture, Food and Rural Development (contract number: 00/R&D/C/53) and Heineken Ireland Ltd. Wort used in this study was kindly provided by Liam Reddy and Declan Goode of the Department of Food Science, Technology and Nutrition, University College Cork. We also acknowledge the technical assistance of Sinead Geary and Linda Walsh of the National Food Biotechnology Centre, Cork.

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(Manuscript accepted for publication April 2002)