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ORIGINAL ARTICLE

Lactic acid bacteria with potential to eliminate fungal spoilage in foods

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Abstract

Aims: To investigate antifungal activity produced by lactic acid bacteria (LAB) isolated from malted cereals and to determine if such LAB have the capacity to prevent fungal growth in a particular food model system.

Methods and Results: The effect of pH, temperature and carbon source on production of antifungal activity by four LAB was determined. *Pediococcus pentosaceus* was used to conduct a trial to determine if it is feasible to eliminate *Penicillium expansum*, the mould responsible for apple rot, using an apple model. *Penicillium expansum* was incapable of growth during the trial on apple-based agar plates inoculated with the antifungal-producing culture, whereas the mould did grow on apple plates inoculated with an LAB possessing no antifungal activity.

Conclusion: Partial characterization of the antifungal compounds indicates that their activity is likely to be because of production of antifungal peptides. The trial conducted showed that the antifungal culture has the ability to prevent growth of the mould involved in apple spoilage, using apples as a model.

Significance and Impact of the study: The ability of an LAB to prevent growth of *Pen. expansum* using the apple model suggests that these antifungal LAB have potential applications in the food industry to prevent fungal spoilage of food.

Introduction

Fungal spoilage of food and feed is a common and global phenomenon. It has been estimated that 5–10% of the world's food production is lost as a result of fungal spoilage (Pitt and Hocking 1999). In addition to the negative financial consequences, fungal spoilage of food and feed also poses a serious health concern. Fungal growth on foodstuffs can result in the production of mycotoxins which are known to be toxic to humans and animals (Sweeney and Dobson 1998; Kabak *et al.* 2006). The ability of fungi to grow in food and feed depends on a variety of factors including water activity (a_w), pH and nutrient availability. In addition, storage conditions as well as the presence of other microbes dictate which types of fungi will grow in a given food system (Monteville and Matthews 2001).

Moulds have the ability to grow in a wide variety of foods, with different genera showing affinity for particular food types. *Fusarium* species, e.g. are notorious for spoilage of grain and cereal plants in the field. Fusaria are known for their production of mycotoxins, which include zearalenone and trichothecenes, such as deoxynivelenol (DON). These toxins are secondary metabolites and are thought to result in immunosuppression as well as carcinogenesis in mammals (Chu and Li 1994; Alves *et al.* 2000; Berek *et al.* 2001). In beer brewing it is believed that production of DON by barley-associated *Fusarium culmorum* and *Fusarium graminearum* strains gives rise to a phenomenon called gushing, described as the spontaneous overfoaming of beer upon opening of the packaged product (Schwartz *et al.* 1995), which means that toxins may survive the malting process and end up in the finished product. Postharvest, stored cereals are frequently

contaminated with *Aspergillus* and *Penicillium* species (Filtenborg *et al.* 1996). These moulds are known to produce mycotoxins which may ultimately carry over into the finished product. For this reason these organisms constitute a realistic health hazard to the brewing, breakfast cereal and baking industry (Legan 1993; Araguás *et al.* 2005).

Spoilage of many other foods has been attributed to fungal contamination, e.g. citrus, pomaceous and stone fruits. The main offenders in the spoilage of these fruits belong to the genera *Penicillium* and include *Penicillium expansum, Penicillium digitatum* and *Penicillium ulaiense* (Mari *et al.* 2003; Plaza *et al.* 2004; Kinay *et al.* 2005). *Penicillium expansum* is known to cause rot in fruits like apples and pears, and has also gained notoriety for its production of the mycotoxins patulin and citrinin (Martins *et al.* 2002). Yeasts have also been implicated in fruit spoilage, with *Rhodotorula* spp. found to be quite prominent in such incidents. A study of fruit salads found *Rhodotorula* spp. to be present on a variety of fruits such as strawberries as well as cantaloupe and honeydew melon varieties (Tournas *et al.* 2006). Other spoilage yeasts present on fruit and in fruit juices include *Zygosaccharomyces* spp. and *Candida* spp. (Casey and Dobson 2004).

Dairy products, including milk and cheese, are also susceptible to fungal growth, leading to deterioration, although certain moulds play an important role in cheese-production. Species of *Penicillium*, including *Penicillium commune* and *Penicillium solitum*, have been found to spoil different varieties of cheese. Thread mould is a frequent problem that arises during Cheddar cheese production, generally caused by moulds belonging to the genera *Phoma*, *Penicillium* and *Cladosporium* (Basílico *et al.* 2001).

With consumer preference for naturally produced foods at an all time high, the need for minimal processing and natural preservation methods is obvious. Lactic acid bacteria (LAB) are naturally occurring in many food systems (Vaughan *et al.* 2001; Marilley and Casey 2004; Tamminen *et al.* 2004; Mitra *et al.* 2005), and have been used for centuries for their fermentation and preservative properties. In particular, their ability to produce antibacterial peptides or bacteriocins has received a lot of scientific attention (Diep and Nes 2002; Cotter *et al.* 2005; Drider *et al.* 2006), while they also sometimes exhibit antifungal activity (Laitila *et al.* 2002; Magnusson *et al.* 2003). Several low molecular weight compounds have been isolated with the capacity to eliminate fungal growth either on their own or synergistically, including organic acids (Cabo *et al.* 2002; Lavermicocca *et al.* 2003; Lind *et al.* 2007), reuterin (Talarico *et al.* 1988), fatty acids (Sjögren *et al.* 2003), proteinaceous compounds (Magnusson and Schnürer 2001) and cyclic dipeptides (Ström *et al.* 2002).

The objective of this study was to identify and investigate antifungal activity of LAB from malted cereals, and to determine if antifungal-producing LAB have the capacity to prevent fungal growth in a particular food model system.

Materials and methods

Cultures and media

LAB strains were cultured in de Mann Rogosa Sharpe (MRS) broth (Oxoid Ltd., Basingstoke, UK) for 48–72 h at 30°C under anaerobic conditions using Anaerocult A gas packs (Merck, Darmstadt, Germany) and stored long-term at −80°C in 40% glycerol. Cultures were stored on MRS agar (Oxoid) for short-term purposes.

Fungal cells or conidial suspensions were stored at −80°C in 40% glycerol and subsequently grown on malt extract agar (MEA; Difco Laboratories, Detroit, Michigan, USA) or potato dextrose agar (PDA; Difco) plates at 25°C for 7 days until sporulation occurred. Spore suspensions were prepared in Ringer's quarter strength sterile solution with 0·8% Tween 80 (Sigma-Aldrich, Schnelldorf, Germany). Yeast strains were grown overnight in malt extract broth with agitation at 25°C or 30°C.

Table 1 shows the fungal strains used in this study and the sources from which they were obtained. The fungi were procured from DSMZ (German Collection for Microorganisms and Cell Cultures), the Department of Microbiology, University of Agricultural Sciences, Uppsala, Sweden, and Department of Microbiology, University College Cork, Cork, Ireland.

Detection of antimicrobial activity and speciation of antifungal LAB

The LAB isolates were originally obtained from red and white sorghum samples sourced from grain stores in South Africa or from wheat and barley samples procured in Iran. The cultures were assayed for production of antifungal compounds using the overlay method (Mayr-Harting *et al.* 1972). LAB cultures were grown for 48 h in MRS broth before being spotted in 5 μl quantities on MRS agar plates. The plates were incubated anaerobically at 30°C for 48 h. Following this, the plates were overlaid with a range of fungal indicators (10^6 spores per cell ml^{-1}) seeded in malt extract agar. The plates were incubated at 25°C or 30°C for 24–48 h, and subsequently examined for inhibitory activity against the fungal indicator. The LAB isolates were also tested for putative bacteriocin-producing activity against the bacteriocin-sensitive indicator *Lactobacillus sake* 2313, according to the method described by Mayr-Harting *et al.* (1972).

Table 1 Inhibitory spectra of antifungal-producing lactic acid bacteria

	Source	CM8	I5	R47	R16
Moulds					
Penicillium notatum	UCC	+++	++	+++	++
Penicillium commune DSM2211	DSMZ	++	++	++	++
Penicillium expansum DSM62841	DSMZ	++	++	+++	+++
Penicillium digitatum DSM2738	DSMZ	++	+	+++	+++
Penicillium verrucosum DSM1836	DSMZ	+	+	+	+
Penicillium roqueforti J268	Sweden	++	++	++	+
Aspergillus nidulans J283	Sweden	++	++	++	++
Yeasts					
Debaryomyces hansenii DSM70590	DSMZ	+	+	+	+
Zygosaccharomyces bailii	UCC	–	–	–	–
Pichia anomala J121	Sweden	–	–	–	–
Saccharomyces cerevisiae DSM70416	DSMZ	–	–	–	–
Saccharomyces diastaticus BSH1	Brewery	–	–	–	–
Rhodotorula mucilaginosa J350	Sweden	+++	++	++	++
Rhodotorula glutinis	UCC	++	++	++	++
Candida krusei	UCC	–	–	–	–

*+++, >10 mm zone of inhibition; ++, 6–10 mm zone of inhibition; +, 1–5 mm zone of inhibition.
DSMZ, German Collection for Microorganisms and Cell Cultures; UCC, University College Cork.

To speciate antifungal-producing LAB, chromosomal DNA was extracted according to the method of Sambrook *et al.* (1989) and used as a template for the amplification of a large portion of the 16S rRNA gene by polymerase chain reaction (PCR) as described by Corsetti *et al.* (2004). The sequences of the obtained PCR products were used to perform a database search against the nonredundant DNA sequence database present at the NCBI website located at http://www.ncbi.nlm.nih.gov/BLAST.

Attempted extraction of antifungal agents from culture medium

The four LAB cultures were spotted in quadruplicate in 5 μl quantities on MRS agar. The plates were incubated anaerobically at 30°C for 48 h, after which time the area surrounding the LAB colony was excised from the agar (15 mm in diameter). This was homogenized in 5 ml of distilled water using a stomacher machine followed by centrifugation at 4293***g*** for 10 min. At this point the aqueous phase of the mixture was removed and the solid phase was discarded. Approximately 4 ml of the aqueous phase was recovered and subsequently tested for antifungal activity.

Adaptations of this procedure were carried out by homogenizing the agar in: (i) 5 ml of 2 mol l^{-1} HCl and (ii) 5 ml of 70%, 80% and 90% ethanol, instead of distilled water.

An overnight culture of each of the antifungal-producing LAB strains was centrifuged to retrieve cell-free supernatant (CFS) and ammonium sulfate was added slowly to give 80% saturation. The mixture was then centrifuged at 4293 ***g*** for 30 min and the precipitate was resuspended in 20 mmol l^{-1} sodium phosphate buffer (pH 6) which was tested for antifungal activity.

Preparation of concentrated CFS

The four antifungal-producing LAB cultures were grown at 30°C for 48 h following which they were centrifuged at 9660 ***g*** for 15 mins and filter-sterilized (0·45-μm pore size; Millipore, Massachusetts, USA) to remove cells. The CFS were then freeze-dried overnight and resuspended in 10 mmol l^{-1} acetic acid (HAc) to give 5-, 10-, 15- and 20-fold concentrated CFS solutions relative to the initial volume of CFS. The CFS from *L. plantarum* BSH2 was concentrated in the same way and acted as a negative control. The samples were tested for activity using the agar overlay method against *Pen. notatum*.

Effect of heat and enzymes on the antifungal activity of the concentrated CFS

The 20-fold concentrated CFS from the four antifungal-producing isolates was subjected to heat treatment at 80°C for 1 h in a water bath and 121°C for 15 min in an autoclave.

The 20-fold concentrated culture filtrates were tested against the proteolytic enzyme proteinase K (Sigma). The enzyme (10 mg ml^{-1} of 20 mmol l^{-1} sodium phosphate buffer, pH 7) and culture filtrates were incubated at 37°C before being tested against *Pen. notatum*. A control sample which contained only 20-fold concentrated CFS and 10 mg ml^{-1} of sodium phosphate buffer (pH 7) was also incubated under the same conditions and tested for activity.

HPLC analysis of lactic acid and acetic acid in culture filtrates

The LAB to be tested were grown in MRS broth to OD_{540} = 2·6 followed by centrifugation and filter-sterilization

(0·45-μm pore size; Millipore) to retrieve CFS which was analysed by high-performance liquid chromatography (HPLC). The analysis was performed using an LKB Bromma, 2150 HPLC system with a refractive index detector, Shodex R1-71 and a Highchrom heating block. A REZEX ROA 8μ H_2 + organic acid column 300 × 7·8 mm (Phenomenex, CA, USA) was used at 65°C with 0·01 N H_2SO_4 as the mobile phase, at a flow rate of 0·6 ml min^{-1}.

Effect of varying growth conditions on production of antifungal compounds

Temperature

The four antifungal-producing isolates were grown at 30°C for 48 h before being subcultured into fresh 10-ml aliquots of MRS broth. These were then incubated for 48 h at six different temperatures: 10°, 21°, 25°, 30°, 37° and 42°C. The cultures were spotted on MRS agar and incubated for 48 h anaerobically at temperatures corresponding to their earlier incubation. The plates were then overlaid with *Pen. notatum* (10^6 spores $ml^{-1)}$ in malt extract agar (0·75%), to which chloramphenicol (Sigma) (10 μg ml^{-1}) was added to halt further bacterial growth and consequent antifungal production of the LAB cultures. The plates were incubated for 48 h at 30°C and then examined for inhibition of fungal growth.

pH value

MRS broth was aliquoted and adjusted to a range of pH values from pH 4 to pH 8 using 2 N HCl or 2 N NaOH. The four LAB isolates were each inoculated into media at each pH interval and incubated for 48 h at 30°C before being spotted on the corresponding pH-adjusted MRS agar plate. The plates were overlaid with malt extract seeded with *Pen. notatum* and containing chloramphenicol as mentioned before.

Carbon source

BSM (bacteriocin screening medium; Tichaczek *et al.* 1992) was used to test if different sugars, as the sole carbon and energy source, would have an effect on the ability of the strains to produce antifungal compounds. This growth medium contains the same ingredients as MRS with the pH set at 6·2 and different sugars can be tested. All four antifungal-producing LAB were inoculated into BSM broth containing one of the following sugars: glucose, maltose, sucrose, fructose, lactose, mannitol or sorbitol. Following incubation for 48 h, the four cultures were spotted on BSM agar containing the particular carbon source in which they had previously been grown. The plates were incubated for 48 h at 30°C anaerobically before being overlaid with *Pen. notatum* as mentioned before.

Application of antifungal-producing LAB in foods

To determine if the antifungal-producing LAB had a potential application in the food industry, a food trial was undertaken using apples as a model. For this purpose, apples were peeled and finely chopped before being weighed. Each batch, contained 200 g of apples in 50 ml of water, was then autoclaved at 115°C for 10 min. While the apple emulsion was still warm it was added to 2% agar before being poured into petri dishes. The apple plates were stored at 4°C for the duration of the trial to limit oxidization.

For the purpose of the trial, *Pediococcus pentosaceus* was chosen as the antifungal-producing LAB on the basis of its inhibitory activity against the fruit-spoilage mould, *Pen. expansum*. *Pediococcus pentosaceus* was either spotted in 5-μl aliquots at various points on the apple plates or spread-plated from an overnight culture on the surface of the apple agar. Another culture, *L. plantarum* BSH2, which does not exhibit antifungal activity, was also spotted or spread-plated onto apple agar plates. Other plates remained uninoculated and acted as positive controls. The trial was set up in triplicate and all plates were incubated for 48 h anaerobically. The plates were overlaid with *Pen. expansum* (10^6 spores ml^{-1}) in 10 ml of 0·75% agar. The plates were incubated at 30°C and examined each day for a period of 14 days.

Results

Identification and speciation of antifungal LAB from cereal samples

Exactly 425 cereal-derived LAB (Hartnett *et al.* 2002) were screened for antifungal activity against the indicator *Fusarium oxysporum*. Of these, four LAB strains were observed to have consistent antifungal activity. The antifungal activity could not be visualized on plates after 24 h, but only after 48 h of incubation. This is consistent with other reports of antifungal activity (Magnusson *et al.* 2003), indicating that the inhibitory agents are secondary metabolites. The four antifungal-producing LAB showed clean, defined zones of inhibition of the fungal indicators (e.g. see Fig. 1). The four LAB cultures did not exhibit antibacterial activity against *L. sake* 2313 (data not shown), and therefore did not appear to produce (Fig. 1a) bacteriocin(s). From 16S rRNA analysis, the four antifungal-producing LAB were identified as *L. plantarum* isolate CM8, *Weissella confusa* isolate I5, *Ped. pentosaceus* isolate R47 and *Weissella cibaria* isolate R16.

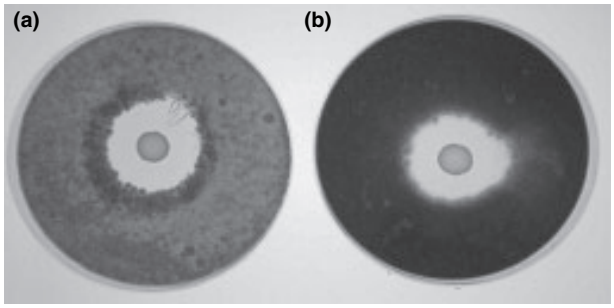

Figure 1 (a) Spot assay with *Pediococcus pentosaceus* R47 on de Mann Rogosa Sharpe (MRS) agar overlaid with *Penicillium notatum* and (b) *Ped. pentosaceus* R47 on MRS agar overlaid with *Penicillium expansum.*

Inhibitory spectra of antifungal-producing LAB cultures

The four antifungal-producing LAB were tested against a selection of yeasts and moulds to determine the range of their inhibitory activity. The fungal indicators were chosen on the basis of their involvement in spoilage of a variety of foods. Table 1 shows the inhibitory spectrum of the four LAB cultures and the extent of their activity towards various fungal strains. The results show that the four antifungal-producing LAB cultures have a similar inhibitory spectrum, and appear to be more directed against moulds rather than yeast.

Extraction of antifungal agents from culture medium

Attempts to extract and concentrate the antifungal compound(s) produced by the four LAB isolates from MRS agar and broth proved unsuccessful and no inhibitory activity could be detected from solid or liquid growth medium.

Another method to concentrate the antifungal compounds in broth, whereby the freeze-dried CFS were resuspended in 10 mmol l^{-1} acetic acid proved to be successful, but only in the 15- and 20-fold concentrated samples. The control, 10 mmol l^{-1} acetic acid concentrated in broth, did not cause any inhibition of the indicator. *Lactobacillus plantarum* BSH2 acted as a negative control and its concentrated CFS was not shown to have inhibitory activity against the fungal indicator.

Effect of temperature and enzymes on antifungal activity

As antifungal activity could not be detected in unconcentrated CFS from the producing LAB, CFS was concentrated to test the effects of temperature and proteolytic enzymes on the antifungal activity. Concentrated supernatant was heated to 80°C for 1 h and also subjected to autoclaving at 121°C for 15 min. However, these treatments did not have any noticeable effect on the inhibitory activity of the four antifungal-producing LAB when tested against the indicator *Pen. notatum*.

When the concentrated supernatants were tested for their sensitivity to the proteolytic enzyme proteinase K, antifungal activity was essentially lost as compared with the original activity. The control sample, containing concentrated CFS with no added enzyme, was unaffected and exhibited the same inhibitory spectrum as the untreated sample. This leads us to assume that the compound(s) responsible for antifungal activity produced by these four LAB are (partly) proteinaceous.

HPLC analysis of CFS

To determine if the observed inhibition of fungal strains by the LAB isolates was attributed to the production of organic acids, HPLC analysis of the CFS from the antifungal-producing LAB was undertaken. *Lactobacillus plantarum* BSH2, which showed no inhibitory activity towards fungal strains, was also subjected to the same analysis. The results confirmed that while substantial amounts of lactate were present in the CFS of the four LAB strains with antifungal activity, a similar amount was observed for the negative control. Table 5 reveals that *L. plantarum* BSH2 produced higher yields of lactic acid, *c.* 181 mmol l^{-1} lactate, as compared with two of the LAB with antifungal activity, i.e. *W. confusa* I5 and *W. cibaria* R16, at 123 mmol l^{-1} and 178 mmol l^{-1} lactate, respectively. The levels of acetate in the CFS were also investigated, with MRS (Oxoid) found to already contain *c.* 30 mmol l^{-1} acetate. This taken into consideration, *L. plantarum* CM8 and *W. confusa* I5 were found to produce concentrations of 60 mmol l^{-1} and 53 mmol l^{-1} of acetate into the CFS, with *Ped. pentosaceus* R47 and *W. cibaria* R16 producing higher levels, i.e. 69 mmol l^{-1} and 72 mmol l^{-1} of acetate, respectively. The negative control, *L. plantarum* BSH2, which did not exhibit antifungal activity, was found to produce even higher levels of acetate at 121 mmol l^{-1}.

Effect of varying growth conditions on production of antifungal compounds

The four LAB were tested to determine whether the strains have the ability to produce their inhibitory activity at a broad range of temperatures, the results of which are presented in Table 2. The cultures were unable to grow at 10° and 42°C, and consequently no antifungal activity could be detected under these conditions. The four cultures grew well when incubated between 21° and 37°C, displaying different levels of antifungal activity, which showed that temperatures between 25° and 30°C lead to optimal production of the antifungal compounds.

Table 2 Effect of temperature on antifungal activity (against *Penicillium notatum*)

Temperature	CM8	I5	R47	R16
10°C	–	–	–	–
21°C	++	+	++	+
25°C	+++	++	+++	++
30°C	+++	++	+++	++
37°C	+	+	+	+
42°C	–	–	–	–

*+++, >10 mm zone of inhibition; ++, 6–10 mm zone of inhibition; +, 1–5 mm zone of inhibition.

Table 4 Effect of carbon source on antifungal activity (against *Penicillium notatum*)

Carbon source	CM8	I5	R47	R16
Glucose	+++	++	+++	++
Sucrose	++	++	++	+
Lactose	+	+	++	+
Maltose	++	++	++	++
Fructose	++	+	++	+
Sorbitol	++	++	+	++

*+++, >10 mm zone of inhibition; ++, 6–10 mm zone of inhibition; +, 1–5 mm zone of inhibition.

The four antifungal-producing LAB were grown in MRS which had been adjusted for pH between 4 and 8. All four LAB isolates grew proficiently at each pH value, although cultures grown at pH 8 appeared slightly less turbid than the other samples. The cultures were tested against the fungal indicator, *Pen. notatum*, after 48 h to determine the effect that varying pH would have on production of antifungal activity. Table 3 shows how each of the four antifungal-producing LAB respond to being grown at various pH values. Antifungal activity appears to be stronger in the lower pH range, although fungal inhibition is still observed when the cultures were grown at pH 8, confirming that the antifungal activity exhibited by these four LAB is not exclusively a result of acid production.

To determine whether there was a correlation between antifungal activity and composition of the growth medium, the four antifungal isolates were grown in a variety of sugars, the results of which are shown in Table 4. The results show that when grown in the different carbon sources antifungal activity is stable, although the amount of activity produced does vary depending on the carbon source.

Application of antifungal-producing LAB in foods

Fungal spoilage of fruit is a major problem worldwide, with *Pen. expansum* known to be the leading cause of blue-rot in apples and pears (Filtenborg *et al.* 1996). Not only do they cause deterioration of the fruit, but they are also known to produce the mycotoxins patulin and citrinin *in situ*. The ability to prevent the growth of *Pen. expansum* on fruit would be of major significance with regards to biopreservation. For this reason, apples were chosen as a model to investigate the potential application of antifungal-producing LAB to eliminate fungal spoilage in food and feed. An apple-based agar growth medium was formulated so that the only available nutrients for the bacteria and mould to utilize would be supplied by the apples (see 'Materials and methods'). *Pediococcus pentosaceus* R47 was chosen as the antifungal-producing culture to challenge the mould in this trial and *L. plantarum* BSH2 acted as a negative control, as it does not exhibit antifungal activity. No mould growth was observed for the 14 days of the trial on the plates containing *Ped. pentosaceus* R47. In contrast to this, uninhibited mould growth was seen on the plates inoculated with *L. plantarum* BSH2 and also on the plates without bacteria. The presence of bacteria on the agar is not sufficient to prevent mould growth, as was observed with the negative control. White mycelia were observed on the negative control and uninoculated control plates at day 5, but not on the plate inoculated with the antifungal-producing strain *Ped. pentosaceus*. By day 14, mould growth, identified by the presence of blue spores, could still not be detected on the plate inoculated with *Ped. pentosaceus* R47, while *Pen. expansum* had continued to grow on the control plates.

Table 3 Effect of varying pH on antifungal activity (against *Penicillium notatum*)

pH	CM8	I5	R47	R16
4	+++	++	+++	++
5	++	++	+++	++
6	++	++	+++	++
7	++	++	++	++
8	++	++	++	++

*+++, >10 mm zone of inhibition; ++, 6–10 mm zone of inhibition; +, 1–5 mm zone of inhibition.

Discussion

LAB have been exploited for decades for their antibacterial activity (O'Sullivan *et al.* 2002). More recently, they have also received scientific attention because of their antifungal potential (Schnürer and Magnusson 2005; Dal Bello *et al.* 2007). To date, the chemical nature of only a handful of these compounds has been elucidated, and much work needs to be performed to fully understand

the biosynthetic pathways and mode of action of fungal inhibitors observed in LAB.

Four cultures with antifungal activity were isolated from *c.* 425 LAB, all of which had originally been isolated from cereals. Identification of the antifungal-producing cultures using 16S rRNA analysis showed that two isolates represent strains of *L. plantarum* (CM8) and *Ped. pentosaceus* (R47). Other environmental isolates belonging to these two species of LAB have previously been found to have potent antifungal activity (Magnusson *et al.* 2003). However, to the best of our knowledge, this is the first time that members of the genus *Weissella* have been linked to the production of antifungal compounds.

Analysis of the organic acid levels in the supernatant (Table 5) leads us to believe that other metabolites, perhaps aside from these organic acids, are responsible for inhibition of the fungal indicators used in this study. This phenomenon was also observed by Magnusson *et al.* (2003), who found that nonantifungal LAB produced organic acids in amounts that were equal to or higher than those produced by LAB with antifungal activity. Purification of antifungal compounds from various LAB has shown that antifungal activity is because of the production of cyclic dipeptides (Ström *et al.* 2002; Magnusson *et al.* 2003). The fact that the antifungal compounds in the current study were found to be protease-sensitive indicates that these LAB may be producing compounds that are proteinaceous in nature, perhaps (cyclic) peptides, similar to those found in other studies. Purification of the compounds by solid-phase extraction (SPE) (Ström *et al.* 2002) will be vital in determining the chemical nature of the inhibition displayed by the four antifungal-producing LAB.

By varying the growth conditions and media in which the four producing cultures were grown, it was observed that the production of antifungal activity is fairly consistent and stable. These antifungal-producing LAB may therefore be good candidates for use in the food industry, also given that LAB are considered GRAS (generally regarded as safe) organisms (Salminen *et al.* 1998), represent natural cereal isolates and can inhibit growth of food-spoilage fungi. The ability to control growth of moulds in food and feed would be beneficial, not only to prevent food spoilage but also to eliminate mycotoxin production (Miura *et al.* 1993; Gökmen and Acar 1998). We successfully showed that it is possible to use one of these strains to prevent growth of spoilage fungi in food, using apples as a model system. To our knowledge, this is the first report of antifungal LAB being applied to improve the microbiological stability of a particular fruit. It is also noteworthy that the inoculum level used (10^6 spores ml^{-1}) for the trial is likely to be much higher than would be expected under natural circumstances, which is a good indication of the inhibitory potential. Although the flesh of the apple was used for our experiment, we would be interested to determine if these antifungal-producing LAB can be applied to the skin of apples and other fruits to prevent fungal growth on the surface. This would be considerably advantageous in the cider industry for example, where problems with fungal spoilage are encountered as the microbes gain entry to the brewing process via the raw material (Jackson *et al.* 2003). The same may also apply to the wine-making industry, where spraying grapes with antifungal-producing LAB may inhibit *Pen. expansum* and thus prevent patulin production (Serra *et al.* 2006). This would also eliminate the need to use fungicides to prevent fungal spoilage of these fruits, hopefully leading to a safer product.

Another potential application of these LAB would be to use them as nonstarter LAB (NSLAB) strains or as adjuncts in cheese-making. During cheese production the cultures may produce the antifungal compounds *in situ*, inhibiting e.g. *Pen. commune,* a mould that is one of the leading causes of cheese deterioration (Basílico *et al.* 2001; Kure *et al.* 2004). Sensory analysis, however, would be required to determine whether the antifungal compounds would impart undesirable flavours on the food.

Table 5 High-performance liquid chromatographic analysis of cell-free supernatant from antifungal-producing lactic acid bacteria and *Lactobacillus plantarum* control

Acid concentrations	CM8	I5	R47	R16	BSH2
Lactate (mmol l^{-1})	185	123	183	178	181
Acetate (mmol l^{-1})	60	53	69	72	121

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