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Antifungal activities of two *Lactobacillus plantarum* strains against *Fusarium* moulds *in vitro* and in malting of barley

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Aims: The *Lactobacillus plantarum* strains VTT E-78076 (E76) and VTT E-79098 (E98) were studied for their antifungal potential against *Fusarium* species.
Methods and Results: *In vitro* screening with automated turbidometry as well as direct and indirect impedimetric methods clearly showed *Lact. plantarum* cell-free extracts to be effective against *Fusarium* species including *Fusarium avenaceum*, *F. culmorum*, *F. graminearum and F. oxysporum*. However, great variation in growth inhibition was observed between different *Fusarium* species and even between strains. The antifungal potential of *Lact. plantarum* E76 culture, including cells and spent medium, was also examined in laboratory-scale malting with naturally contaminated two-rowed barley from the crops of 1990–96. The growth of the indigenous *Fusarium* flora was restricted by the addition of *Lact. plantarum* E76 to the steeping water. However, the antifungal effect was greatly dependent on the contamination level and the fungal species/strains present on barley in different years.
Conclusions: *Lactobacillus plantarum* strains E76 and E98 had a fungistatic effect against different plant pathogenic, toxigenic and gushing-active *Fusarium* fungi.
Significance and Impact of the Study: The present study indicates that *Lact. plantarum* strains with known and selected characteristics could be used as a natural, food-grade biocontrol agent for management of problems caused by *Fusarium* fungi during germination of cereals.

INTRODUCTION

Fusarium is one of the most important genera of plant pathogenic fungi, with a record of devastating infections in various economically important plants (Chelkowski 1989). In addition to causing yield and quality reduction of cereals, fusaria have caused severe problems for the end users (Steffenson 1998). Certain species of fusaria are capable of producing toxic secondary metabolites, mycotoxins, which can be accumulated in the infected plants and stored material. Zearalenone and various trichothecenes, such as deoxynivelenol (DON), can be detected in cereals such as maize, rice and barley (Chelkowski 1989). It has been reported that cereal-based products are major sources of intake of these toxins (Eriksen and Alexander 1998).

The most prominent DON-producing species are *Fusarium graminearum* (teleomorph *Gibberella zeae*) and *F. culmorum*. These strains are known to strike barley production areas in North America, Europe and Asia. Fungal growth and production of secondary metabolites are dictated by many factors, such as crop susceptibility, agricultural practices, climate, harvesting, storage and process conditions (Chelkowski 1991; Schildbach 1995; Steffenson 1998). During the years 1993–98 in North America, the widespread occurrence of *Fusarium* head blight (also called scab) and subsequent production of DON in barley was associated with a high incidence of *F. graminearum* (Schwarz *et al.* 1995a, 1995b). In addition to grain quality deterioration, Schwarz *et al.* (1995a) and Scott *et al.* (1993) showed that *Fusarium* toxins could be produced during the malting process of barley and be transferred into the finished beer.

In addition to being potential mycotoxin producers, *F. graminearum* and *F. culmorum* moulds are known to be active gushing inducers. Gushing is a term used to describe spontaneous overfoaming of beer on the opening of the packaged product and it is often associated with heavy *Fusarium* infection of barley or malt (Amaha and Kitabatake 1981; Schwarz *et al.* 1996). Gushing is a very complex phenomenon which can, at least partially, be explained by the

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secretion of specific factors by fungi in barley in the field, during storage or during the malting process (Kleemola *et al*. 2001; Munar and Sebree 1997; Amaha and Kitabatake 1981; Haikara *et al*. 2000).

Some chemical fungicides are generally effective in controlling the growth of harmful fungi but, nowadays, the use of chemicals is considered undesirable because of concerns over residues. As a consequence, research has been directed towards developing more natural means of prevention of fungal contamination. Lactic acid bacteria (LAB) are widely used in food and feed fermentation, contributing to the safety, stability, flavour and structure of the products. The antibacterial effects of LAB have been extensively studied (Stiles 1996; Salminen and von Wright 1998). The microbistatic and/or microbicidic action of LAB is based on both competition for nutrients and the production of various antimicrobial compounds, such as organic acids, hydrogen peroxide, bacteriocins and low molecular weight antimicrobial agents (Ouwehand 1998).

Several investigations have been conducted to examine the antifungal properties of LAB against fungal contaminants in dairy and sourdough baked products (El-Gendy and Marth 1981; Batish *et al*. 1989; Batish *et al.* 1990; Suzuki *et al*. 1991; Gourama and Bullerman 1995a; Roy *et al*. 1996; Batish *et al.* 1997; Gourama 1997; Corsetti *et al*. 1998; El-Nezami and Ahokas 1998; Gobbetti 1998; Stiles *et al*. 1999; Lavermicocca *et al*. 2000). Furthermore, it has been shown that certain LAB, such as a dairy strain *Lactococcus lactis*, as well as *Lactobacillus and Pediococcus* meat starters, silage mixtures containing *Lact. acidophilus*, *Lact. bulgaricus* and *Lact. plantarum* species, and probiotic *Lact. rhamnosus* strains either suppressed mycotoxin biosynthesis or effectively removed preformed mycotoxins (Coallier-Ascah and Idziak 1985; Luchese *et al.* 1992; Gourama and Bullerman 1995b, 1997; El-Nezami and Ahokas 1998; El-Nezami *et al.* 1998a, 1998b; Haskard *et al*. 2001).

Malting is a complex biological process in which the germination of barley leads to the synthesis of hydrolytic enzymes and degradation of grain structure (Bamforth and Barcley 1993). Malted (germinated) barley is then the main raw material in beer brewing. In addition, malt can be used for the production of distilled spirits or can be processed into extracts used by different branches of the food industry. The malting conditions favour intensive microbial growth. It is well known that multitudes of microbes present on and in barley kernels greatly influence the quality of malt and beer (Flannigan 1996; Noots *et al.* 1998). Earlier studies at VTT Biotechnology revealed a group of LAB with antagonistic activity against Gram-negative bacteria and fungi. This gave rise to an idea for a new application in which LAB could be used as protective cultures in the malting process for restricting the growth of harmful microbes such as *Fusarium* moulds (Haikara *et al*. 1993; Haikara and Mattila-Sandholm 1994).

In the present study we examined the antifungal potential of two *Lact. plantarum* strains, E76 and E98, against different plant pathogenic, toxigenic and gushing-active *Fusarium* fungi. The antifungal activities of these lactobacilli against selected *Fusarium* strains were studied *in vitro* with an automated turbidometer and impedimetric methods. In most of the investigations hitherto conducted, the antifungal activity of LAB has been demonstrated in bioassays with fungi cultivated in defined medium on agar plates or in liquid cultures. We studied the effects of *Lact. plantarum* strains on *Fusarium* growth on barley kernels. This paper also describes the ability of LAB to restrict the growth of *Fusarium* flora in laboratory scale malting of naturally contaminated barley.

MATERIALS AND METHODS

Fungal cultures and *Lactobacillus plantarum* strains

The *Fusarium* cultures, including 20 strains of the species *F. graminearum* (teleomorph *G. zeae*), *F. culmorum*, *F. avenaceum* (teleomorph *G. avenacea*) and *F. oxysporum*, were provided by the VTT Culture Collection (Table 1). The cultures were grown at 25°C for 7 d on Potato Dextrose Agar (PDA; Difco Laboratories, Detroit, MI, USA).

Lactobacillus plantarum VTT E-78076 (E76, isolated from beer) and VTT E-79098 (E98, isolated from pickled cabbage) were grown in MRS broth (Oxoid Ltd, Basingstoke, UK) at 30°C for 72 h. To prepare cell-free culture filtrate, bacterial suspension was centrifuged at 3000 *g* for 10 min and the supernatant fluid filtered through a 0·45-μm pore-size filter (Millex-HA; Millipore, S.A., Molsheim, France). The sterile, cell-free filtrate was stored at 4°C. The LAB were enumerated on MRS agar plates (Oxoid) incubated in anaerobic conditions at 30°C for 72 h.

Detection of antifungal activity with automated turbidometry

The *Fusarium* strains were induced to sporulate in 1% CMC broth cultivated with shaking at 25°C for 7 d (Booth 1977). CMC broth contained (g l^{-1}): carboxymethylcellulose, 10; NH_4NO_3, 1; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0·5 and yeast extract, 1. A suspension containing 1 ml *Fusarium* CMC broth culture and 9 ml 0·05% Tween 80 (v/v) was then mixed carefully with glass beads (10–15 per test tube) to break the mycelium. The mycelial debris was removed by filtration through sterile glass wool. The number of conidia was counted microscopically by use of a counting chamber (Thoma, Knittel Gläser, Germany).

An automated turbidometer (Bioscreen®; Labsystems Oy, Helsinki, Finland) was used for measuring the antifungal potential of *Lact. plantarum* strains against *Fusarium* strains;

Table 1 *Fusarium* strains used in the antifungal studies

Species	Strain no.	Code used in the text	Origin	Region
F. graminearum	VTT D-76013	D13	Not known	Finland
(teleomorph	VTT D-82081	D81	Rye grass	Germany
Gibberella zeae)	VTT D-82082	D82	Barley	Finland
	VTT D-82086	D86	Barley	Finland
	VTT D-79129	D129	Cereal grain	Denmark
	VTT D-80136	D136	Cereal grain	Finland
	VTT D-80137	D137	Cereal grain	Finland
	VTT D-80143	D143	Not known	Not known
	VTT D-82169	D169	Barley	Finland
	VTT D-82170	D170	Barley	Finland
	VTT D-82177	D177	Barley	Finland
	VTT D-95470	D470	Maize	USA
	VTT D-95471	D471	Maize	USA
	VTT D-95472	D472	Maize	USA
F. avenaceum	VTT D-80141	D141	Barley	Finland
(teleomorph *G. avenacea*)	VTT D-80147	D147	Barley	Finland
F. culmorum	VTT D-80148	D148	Barley	Finland
	VTT D-80149	D149	Barley	Finland
	VTT D-82171	D171	Barley	Finland
F. oxysporum	VTT D-98690	D690	Plant material	The Netherlands

30 μl of the cell-free supernatant fluid and 30 μl of the test organism in the growth medium were dispensed into microtitre plate wells with 240 μl Potato Dextrose growth medium (PD broth; Difco). The spore suspension was adjusted to 1000 conidia well^{-1}. Unless otherwise stated, in the control wells, the antimicrobial agent was replaced by an equal volume of sterile, unfermented MRS broth without glucose and with the pH adjusted to 3·8 with HCl prior to testing. In addition to cell-free supernatant fluids, *F. avenaceum*, *F. culmorum* and *F. graminearum* strains were screened for potential inhibitory action caused by lactic acid corresponding to the amount produced into the MRS medium; 30 μl 2·5% lactic acid with the pH adjusted to 3·8 with NaOH were dispensed with 240 μl PD broth and 30 μl of the test organism. *Fusarium* strains were incubated in the Bioscreen® with shaking at 25°C for 72 h. All of the determinations were carried out with four replicates and results are expressed as the mean values. The area under the growth curve given by the Bioscreen® was used as a measure of fungal growth and area-reduction percentage values were used to describe the inhibitory effects of the antimicrobial compounds (Skyttä and Mattila-Sandholm 1991).

Detection of antifungal activity with impedimetric measurements

Selected *Fusarium* strains were cultivated on PDA at 25°C for 7 d. The mycelium and spores were collected with swabbing and diluted with 0·05% Tween 80. Glass beads were used to break the mycelium and the debris was removed by filtration through glass wool. Spore densities were determined microscopically by use of a counting chamber (Thoma).

The change in impedance in the growth medium caused by microbial growth was monitored automatically using a BacTrac® 4100 instrument (Sy-Laboratory, Neupurkersdorf, Austria). The instrument consisted of autoclavable glass measuring vessels with two pairs of electrodes connected to a microprocessor. Equal volumes (1 ml) of cell-free culture filtrate and *Fusarium* spore suspension (10^4 spores ml^{-1}) were mixed with 8 ml commercial Yeast and Mould medium (B501; Sy-Laboratory) and incubated at 25°C for 48 h. The growth of micro-organisms changed the concentrations of ions in the growth medium and in the layers surrounding the electrodes. Changes in capacitance (E-value = relative electrode impedance) were monitored. The detection limit (threshold value) was set to 7%. The time needed to reach the threshold level was used as a measure of fungal growth and the delay in reaching the set level was used to describe the inhibitory effects of LAB. Two parallel determinations were carried out with three replicates and the results expressed as average values.

In indirect impedance measurements, autoclaved barley kernels (moisture content after autoclaving 40% (w/w)) were used as a substrate for fungi. Sterile vials were filled with 10 hydrated kernels. Equal volumes (0·5 ml) of the cell-

free culture filtrate and fungal spore suspension (10^5 spores ml^{-1}) were added to the kernels and mixed carefully. The tightly sealed vials were placed into the measurement cells containing 1 ml 1% KOH solution. In the control samples, the antimicrobial agent was replaced with sterile MRS medium. The samples were incubated in the BacTrac 4100 apparatus at 25°C for 4 d. The carbon dioxide produced by the *Fusarium* fungi was absorbed in the 1% KOH solution and the reduction in conductivity of the solution was measured. The chemical reaction between carbon dioxide and the KOH solution caused a decrease in the *M*-value according to the amount of carbon dioxide produced by fusaria. Two parallel determinations were carried out with three replicates and the results expressed as average values.

Laboratory scale malting experiments with naturally infested barley

Lactobacillus plantarum E76 strain was grown in MRS broth (Oxoid) at 30°C for 3 d. Before addition into the malting process, the cultures were stored in a cold room (+ 4°C) overnight. Two-rowed Finnish barley (cultivar Kymppi) was used in the laboratory scale malting experiments. Batches of 1 kg barley were steeped separately for 2 d at 12°C to a moisture level of 45–46% with two alternating wet steep periods (about 7–8 h each) and two air rests (about 16 h each). The LAB culture, including cells and spent medium, was added into the steeping waters at a level of 8% (v/v) of the steeping water (120 ml/1500 ml water). After the steeping, samples were transferred to a micromalting unit (Seeger, C. Seeger, Maschinenfabrik, Stuttgart, Germany) and germinated at 14°C for 6 d. At the end of the germination, the samples were kilned in a kilning unit (Seeger) for 21 h with a stepwise temperature increase from 50 to 85°C (moisture content after kilning was approx. 4%). After kilning the rootlets were removed by sieving.

Samples for mycological analyses were taken from the original barley, barley after steeping and malt after kilning. For *Fusarium* analyses, 100 randomly selected barley kernels were placed on a selective Czapek–Dox agar containing Iprodion and Dichloral (CZID agar) and on a wet filter paper in which the barley kernel itself was the only substrate for fungal growth (Gyllang *et al.* 1981; Abildgren *et al.* 1987). Germination of grains was prevented by wetting the filter paper with 15–20 ml 0·2% 2,4-D-sodium salt solution (2,4-dichlorophenoxy acetic acid). For the analysis of malt, sterile water was used instead of the 2,4-D-sodium solution. The CZID plates were incubated at 25°C for 7 d and filter paper plates at 25°C for 21 d. *Fusarium* moulds were identified under a stereomicroscope on the basis of typical colony form and colours. Identification was confirmed by conidia morphology with a light microscope (magnification 400 ×). The results of the determinations of fungi were expressed as the per cent *Fusarium*-contaminated barley kernels in the total number of kernels.

The number of LAB was determined from the homogenized sample by plating on MRS agar (Oxoid). A sample (10 g) was homogenized with 90 ml sterile saline solution using a laboratory blender (Stomacher 400, Seward Medical, London, UK). Samples were incubated in anaerobic conditions at 30°C for 3–5 d.

Analysis of lactic acid

Lactic acid was determined by HPLC with a u.v. detector at 210 nm and a cation exchanger (Aminex HPX-87; Bio-Rad Laboratories, Hercules, CA, USA). The acids were eluted with 0·003 mol l^{-1} sulphuric acid at 65°C.

RESULTS

Measurement of antifungal activity of *Lactobacillus plantarum* strains E76 and E98 against *Fusarium* fungi by automated turbidometry

Automated turbidometry was used to detect the antifungal potential of cell-free culture filtrates of *Lact. plantarum* E76 and E98 against 20 different plant pathogenic, mycotoxigenic and gushing-active *Fusarium* strains which mainly originated from cereals (Table 1). The inhibitory effects of cell-free extracts on the growth of different *Fusarium* strains are presented in Fig. 1. Both *Lact. plantarum* strains restricted the germination of conidia of most of the *Fusarium* strains. However, great variation in growth inhibition was observed between different *Fusarium* strains, some strains being more resistant to the antimicrobial substances produced by these lactobacilli. In particular, the *F. graminearum* strains D13, D82, D136 and D169 were insensitive to cell-free extracts. The growth of other *F. graminearum* strains was inhibited by 26–54% by both of the *Lact. plantarum* strains. The LAB strains were also effective against *F. avenaceum*, *F. culmorum* and *F. oxysporum* strains. Cell-free extracts of lactobacilli gave rise to 29–38% growth inhibition with *F. avenaceum* strains, 26–41% with *F. culmorum* strains and approx. 50% with one *F. oxysporum* strain. Although the *Lact. plantarum* strains originated from different sources, the two different strains had surprisingly similar antifungal spectra.

Table 2 shows the effects of cell-free culture filtrates compared with the effect of lactic acid, corresponding to the amount of lactic acid produced in MRS broth, against randomly selected *F. avenaceum*, *F. culmorum* and *F. graminearum* strains. After 72 h fermentation, the pH of the *Lact. plantarum* MRS culture supernatant fluid was 3·8 and the concentration of lactic acid was 25–27 g l^{-1}. The results indicated that low pH and lactic acid alone could not explain the inhibitory action of the *Lact. plantarum* strains against

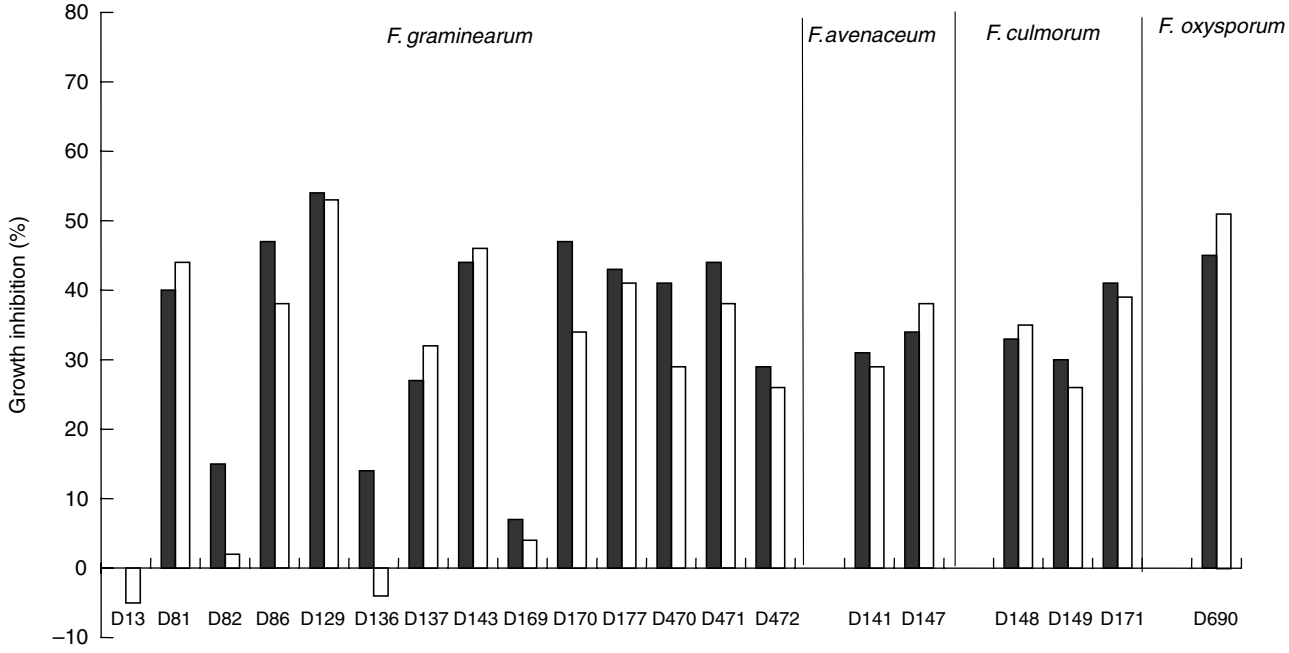

Fig. 1 Growth inhibition (%) of *Fusarium graminearum*, *F. avenaceum*, *F. culmorum* and *F. oxysporum* strains by cell-free culture filtrates of *Lactobacillus plantarum* E76 (■) and E98 (□) as measured by an automated turbidometer (Bioscreen®). Values are means of four replicates. The S.D. between replicate samples ranged from 1 to 2%

Table 2 Growth inhibition (%) of *Fusarium avenaceum* D141 and D147, *F. culmorum* D171 and *F. graminearum* D129 by lactic acid* compared with cell-free supernatant fluids of *Lactobacillus plantarum* E76 and E98

	Growth inhibition (% ± S.D.)		
Fusarium strains	Lactic acid*	*Lact. plantarum* E76	*Lact. plantarum* E98
F. avenaceum D141	20 ± 6	33 ± 1	32 ± 4
F. avenaceum D147	6 ± 3	35 ± 2	30 ± 2
F. culmorum D171	− 2 ± 5	53 ± 1	47 ± 2
F. graminearum D129	− 1 ± 2	49 ± 8	50 ± 5

*The concentration of lactic acid in the well was 0·25% corresponding to the amount of lactic acid added along with the cell-free supernatant fluid of *Lact. plantarum*. In the control wells lactic acid was replaced by an equal volume of sterile growth medium.
Results are the average ± S.D. of four replicate samples.

Fusarium fungi (Table 2). The maximum inhibitory effect of lactic acid was 20% against *F. avenaceum*, whereas the cell-free extracts of *Lact. plantarum* strains inhibited the growth of *Fusarium* strains by 30–50%. Growth of *F. graminearum* D129 and *F. culmorum* D171 strains was not affected by the presence of lactic acid. Acidification of unfermented MRS with HCl to pH 3·8 did not inhibit the germination of *Fusarium* conidia (data not shown).

Effects of cell-free culture filtrates on *Fusarium* metabolism measured by direct and indirect impedimetry

In addition to turbidometric measurements, another *in vitro* method, impedimetry, was used to verify the antagonistic activity of *Lact. plantarum* strains against *Fusarium* fungi. One strain from each *Fusarium* species was selected for direct impedimetric measurements. The metabolic activities of *F. avenaceum* D147, *F. culmorum* D148, *F. graminearum* D470 and *F. oxysporum* D690, in the presence of cell-free culture filtrates, were monitored in commercial Yeast and Mould medium. Figure 2 demonstrates the growth curve of *F. culmorum* D148 obtained from BacTrac® measurements, showing the change in capacitance with time. In control samples the detection time for fungal growth was approx. 18–20 h. As seen from the results presented in Table 3, cell-free culture filtrates of LAB caused a significant delay in detection time. Impedance values did not reach the threshold level (7%) during the 48 h incubation when *Lact. plantarum* E76 or E98 culture filtrates were used.

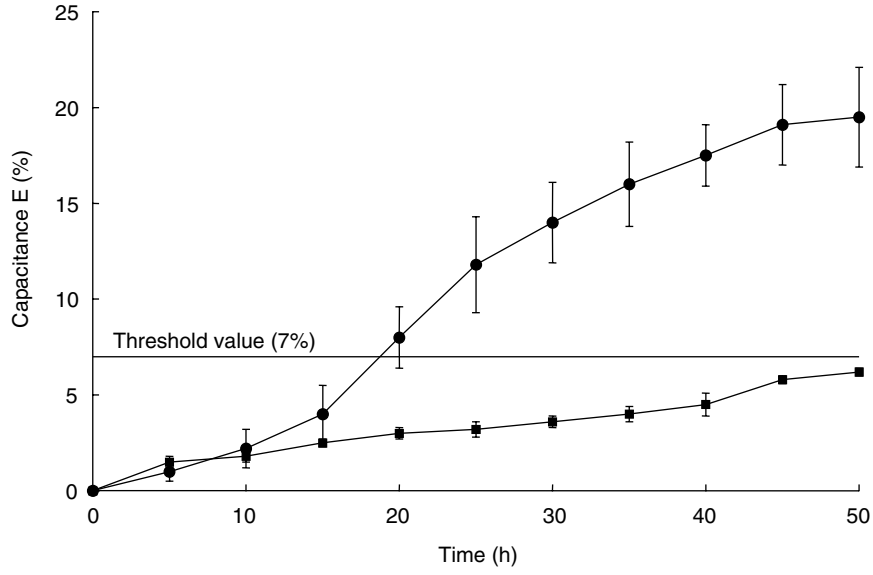

Fig. 2 Antifungal activities of *Lactobacillus plantarum* E76 and E98 culture filtrates (■) against *Fusarium culmorum* D148 (●) measured by a direct impedimetric method. The impedance readings were taken by BacTrac® during 48 h incubation and presented as curves showing the change in the capacitance with time. The detection limit (threshold value) was set to 7%

Table 3 Delay (h) of *Fusarium* growth in the presence of cell-free culture filtrates of *Lactobacillus plantarum* E76 and E98 as measured by a direct impedimetric method

	Delay in the detection time (h)	
	Lact. plantarum E76	*Lact. plantarum* E98
F. avenaceum D147	>48	>48
F. culmorum D148	>48	>48
F. graminearum D471	>48	>48
F. oxysporum D690	>48	>48

Values are means of three replicates from two different impedance tests.

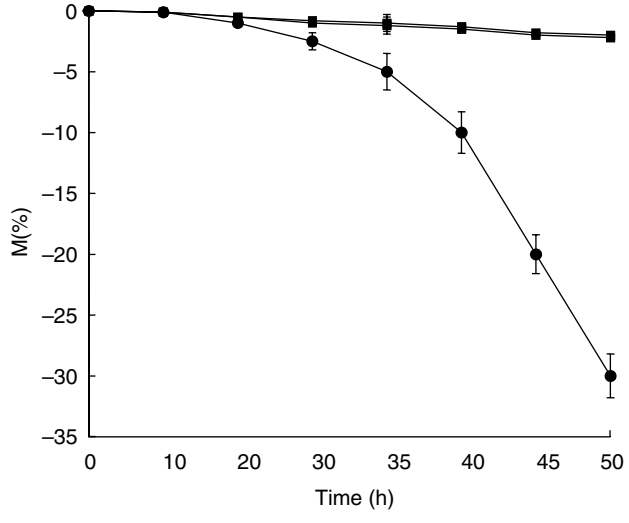

Fig. 3 Carbon dioxide production of *Fusarium culmorum* D148 (●) on hydrated barley kernels in the presence of cell-free culture filtrates of *Lactobacillus plantarum* E76 and E98 (■) as measured by an indirect impedimetric method. The chemical reaction between carbon dioxide and KOH solution caused a decrease in the *M*-value as a function of the amount of carbon dioxide produced by the fungi

The choice of growth medium was critical in direct impedance measurement. Hence, a specific medium was used in order to obtain a usable electrical signal. In the indirect method, the growth medium could be replaced with solid substrates. We used hydrated barley grains (moisture content after autoclaving 40% (w/w) as a medium for *Fusarium* growth. This allowed the monitoring of the fungal growth and potential antifungal activity of LAB in a more natural environment. *Fusarium* fungi proliferated well on barley kernels and intensive production of carbon dioxide was recorded after 30 h of incubation (Fig. 3). *Lactobacillus plantarum* cell-free extracts applied on hydrated barley kernels significantly reduced the metabolic activity of *F. culmorum* D148 as measured by carbon dioxide production during 48 h incubation.

Effect of *Lactobacillus plantarum* E76 on the occurrence of *Fusarium* fungi during laboratory scale malting of naturally contaminated barley

In vitro studies revealed that the growth of fusaria could be restricted by *Lact. plantarum* strains. Most antifungal studies have been carried out in controlled laboratory environments

with pure cultures or artificially contaminated material. The present investigation was carried out to elucidate the antifungal potential of *Lact. plantarum* in the true environment with naturally infested barley. *Lactobacillus plantarum* E76 originated from beer and was, therefore, a better candidate for malting experiments as it was already well adapted to the malting and brewing environment. Figure 4 shows the effects of *Lact. plantarum* E76 on *Fusarium* contamination of barley after the steeping process and in final malt. The data were collected from 43 laboratory scale maltings carried out in 1991–97. *Lactobacillus plantarum* E76 culture broth, including cells, was added to the steeping waters of barley to 8% (v/v) of the steeping water. The number of *Lact. plantarum* E76 in the steeping water and also in the malting barley samples was 10^7–10^8 cfu g^{-1}. Addition of LAB culture into the process reduced the pH of the water to 4·0–4·4 after the first steep and to 3·9–4·2 after the second steep. In control samples, the pH of the water

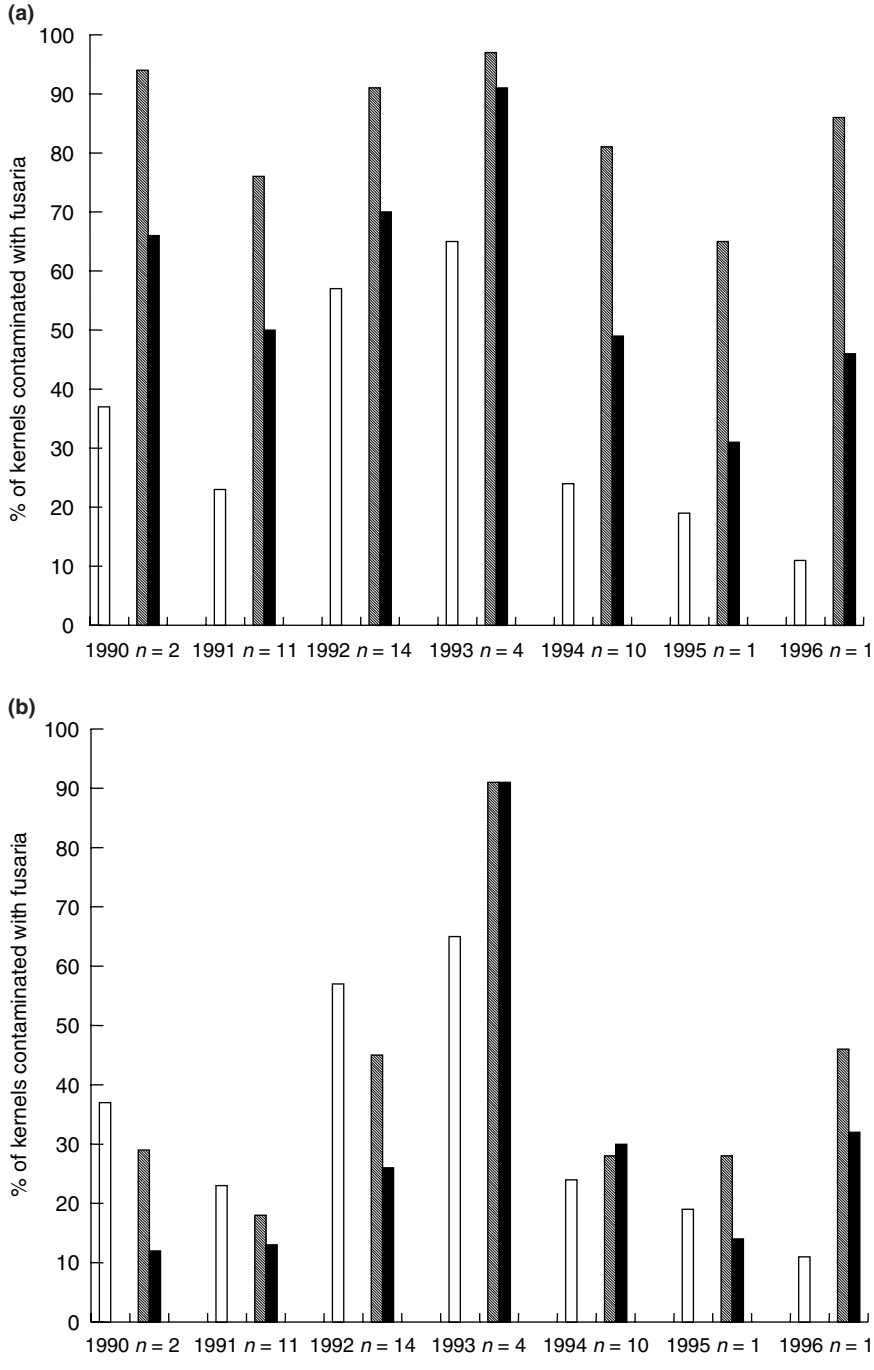

Fig. 4 Effect of *Lactobacillus plantarum* E76 (added to steeping water) on the occurrence of *Fusarium* fungi during laboratory scale maltings of Kymppi barley. The data were collected from different laboratory scale maltings carried out in 1991–97. The counts are mean values obtained from different individual malting experiments. Effects of *Lact. plantarum* E76 on the *Fusarium* contamination of (a) steeped barley and (b) malt. Kernels (%) contaminated with *Fusarium* fungi in native barley (□), control samples (▨) and samples with added *Lact. plantarum* E76 (■)

was 6·8–7·5 after the first steep and 6·4–7·0 after the second steep.

Fusarium counts were determined by two different methods, selective *Fusarium* agar (CZID) and the filter paper method. Due to the similar results obtained from the two determinations, only counts determined with the filter paper method are presented in this paper (Fig. 4). Soaking of kernels induced microbial growth at the beginning of the malting process. In particular, the dormant *Fusarium* conidia activated surprisingly rapidly after the transfer of barley into the steeping water (Fig. 4a). In an optimal growth environment, increases in intracellular ATP levels of macroconidia have been detected after only 15 min incubation (our unpublished results). Intensive *Fusarium* growth was observed during the steeping, even when the original barley had only a low level of *Fusarium* contamination (Fig. 4a). Approximately 30–50% higher *Fusarium* counts were measured after the steeping stage compared with the original contamination of barley. The highest increase recorded was from 11 to 86% of kernels contaminated with *Fusarium* in control samples. Kilning reduced the viable count of fusaria, except in the case of barley of the 1993 crop in which over 90% of kernels were contaminated with *Fusarium* fungi after kilning (Fig. 4b).

Due to the intensive growth of fusaria during the very first hours of steeping, the addition of *Lact. plantarum* E76 in the early stage of malting was important. In the malting experiments carried out with barley of the crops of 1990–92, *Lact. plantarum* E76 diminished *Fusarium* contamination of barley by over 20% (Fig. 4a). Correspondingly, in the final malts, the difference was 5–17% (Fig. 4b). As seen in these figures, rather large variations were evident in the reduction of *Fusarium* contamination. This was obviously due to the differences in the composition of *Fusarium* flora and the contamination level of barley. In 1993, due to the heavy contamination of barley, the effect of *Lact. plantarum* on the growth of *Fusarium* moulds during steeping was limited. On the other hand, a marked reduction (30–50%) was observed in *Fusarium* counts after steeping of barley in malting of barley from the harvest years 1994–96 when *Lact. plantarum* E76 culture was applied.

DISCUSSION

Although *Fusarium* moulds belong to the natural mycoflora of cereals, heavy infection may cause serious problems in the food and feed industry. Contamination of the barley crop by fusaria is of concern particularly in years when bad weather conditions favour the growth of toxigenic and gushing-active *Fusarium* species. Strict control of the incoming barley lots is vitally important in order to reject contaminated material prior to purchasing. However, malting conditions favour significant growth of *Fusarium* fungi, including species which might produce mycotoxins and gushing factors during the process. Therefore, there is a need for efficient and safe ways to control the growth and metabolic activity of fungi in raw materials as well as during the process.

Our results clearly indicated the antifungal potential of two *Lact. plantarum* strains, VTT E-78076 and VTT E-79098, against different plant pathogenic, toxigenic and gushing-active *Fusarium* fungi commonly isolated from cereals, especially barley. The antifungal activity of *Lact. plantarum* strains has also been reported by other investigators (Gourama and Bullerman 1995a; Lavermicocca *et al.* 2000). Karunaratne *et al.* (1990) reported that commercial silage inoculum had antifungal activity against *Aspergillus flavus* subsp. *paraciticus* in liquid culture. However, mould growth was not affected by these silage cultures on solid substrate. On the contrary, an increased level of aflatoxin B_1 was detected in rice in the presence of silage inoculant. Precautions must be taken when selecting LAB for biocontrol agents in food or feed processes, as some investigators have reported stimulation of mould growth and toxin synthesis by LAB (Wiseman and Marth 1981; Luchese and Harrigan 1990).

Most studies have demonstrated the antagonistic activity of LAB *in vitro* against one representative strain of a certain fungal species or genus. In many cases, the antifungal activity of LAB has often been evaluated only with the agar well diffusion method. The present study screened 20 different *Fusarium* strains with automated turbidometry. Our study showed that automated turbidometry was a reliable method for the monitoring of fungal biomass and an excellent tool for screening the antifungal potential of LAB antimicrobial agents against fungal cultures. Such a finding has also been reported by Broekaert *et al.* (1990), Raaska and Mattila-Sandholm (1995) and Laine *et al.* (1996). In addition to turbidometric measurements, direct and indirect impedimetry was used to verify the antifungal activity of *Lact. plantarum* strains against selected *Fusarium* strains. *Lactobacillus plantarum* E76 and E98 cell-free extracts effectively suppressed the metabolic activity of *Fusarium* fungi in commercial Yeast and Mould medium in direct impedence. The great advantage of the indirect over the direct impedimetric method was that solid substrates could also be used as a growth medium (Dezenclos *et al.* 1994). Significant reduction of carbon dioxide production by fungi was observed when barley kernels were used as a solid substrate in indirect impedimetric measurements. With this experiment, we showed that the growth of *Fusarium* fungi could also be inhibited on a solid substrate and not only in liquid cultures in which the diffusion of antimicrobial compounds was far better than in solid substrate.

The antifungal activity of LAB is certainly a complex phenomenon and still partly unknown. Although the nature of the antifungal substances and the mode of action

mechanisms were not studied in the present investigation, our results indicated that the observed inhibitory activity could not be explained by low pH and lactic acid alone. Conidia of *Fusarium* fungi germinated in a very acidic environment even at pH 3 (unpublished data). Several other studies have reported that the antifungal activity of LAB is not only related to the production of organic acids and hydrogen peroxide. Rather, it is a combined effect of several inter-related factors. Only a few studies have reported the production of specific antifungal compounds. In our previous study we identified low molecular mass antimicrobial compounds from the culture filtrate of *Lact. plantarum* E76 (Niku-Paavola *et al.* 1999). Mixtures of compounds, such as benzoic acid, methylhydantoin and mevalonolactone, present in very low concentrations, acted in synergy with lactic acid and had antimicrobial activity against the Gram-negative bacterium *Pantoea agglomerans* and *F. avenaceum.* Roy *et al.* (1996) reported that some of the antifungal inhibitory compounds are proteinaceous in nature. The antifungal activity of a sourdough *Lact. sanfrancisco* strain was due to mixtures of organic acids, such as acetic, caproic, propionic, formic, butyric and n-valeric acids (Corsetti *et al.* 1998). Another common sourdough isolate of *Lact. plantarum* had a broad antifungal spectrum against bread spoilage moulds (Lavermicocca *et al.* 2000). The main causative agents were identified as phenyllactic acid and its corresponding hydroxy derivate. Magnusson and Schnürer (2001) characterized a broad-spectrum, heat-stable antifungal compound produced by the grass silage isolate *Lact. coryniformis* subsp. *coryniformis*. Recently, Okkers *et al.* (1999) reported that a bacteriocin-like peptide, pentocin TV35b, produced by *Lact. pentosus* had a fungistatic effect against *Candida albicans*. *In situ*, in practical applications, the antimicrobial action is often the sum of many factors and, in many cases, not only extracellularly produced compounds but also viable cells are needed for the maximum action. Haikara *et al.* (1993) found that whole cultures of *Lact. plantarum* E76 (cells and spent medium) were needed for the maximal restriction of harmful micro-organisms during malting.

Malting conditions favour intensive microbial growth, which can be regarded as an important part of the malting process. Therefore, microflora management should be conducted in such a way that the growth of beneficial or neutral microbes is favoured by the simultaneous inhibition of harmful micro-organisms. Steeping of barley is a crucial step in the malting process in which bacterial and fungal growth is rapidly activated (Noots *et al.* 1998). This study showed intensive growth of fusaria during the steeping process, even when the original barley had only a low level of *Fusarium* contamination. Therefore, *Lact. plantarum* E76 cultures were added in the early stage of malting. We demonstrated that the growth of the indigenous *Fusarium* flora of barley can be restricted by addition of *Lact. plantarum* E76 into the steeping water of naturally contaminated two-rowed barley. However, our results indicated that the *Fusarium* flora differed in its susceptibility to LAB antimicrobial agents in different years. *In vitro* measurements with 20 different *Fusarium* strains showed that great variation in growth inhibition exists for different *Fusarium* species and even for different strains of the same species.

Alternative methods to control fungal growth during the malting process have recently been published. Boivin and Malanda (1997) reported that the growth of *Fusarium*, *Aspergillus* and *Penicillium* fungi during the malting process was reduced by the yeast-like fungus *Geotrichum candidum* isolated from a malting house. Papadopoulou *et al.* (2000) suggested that fungal proliferation could be restricted by the addition of hop beta-acids into the malting process. Moreover, they demonstrated that the growth of fungi was inhibited by washing of barley first with sodium hypochlorite (alkaline wash) followed by an acid wash with hydrochloric acid. Olkku *et al.* (2000) reported a novel invention in which the mould content of barley was effectively reduced by exposing grains to heat (60–100°C) for 0·5–3 s prior to the malting process. Heat treatment notably decreased the *Fusarium* contamination of barley without affecting grain germination. Moreover, a significant reduction of mycotoxin production and decrease in gushing tendency were observed. Heat treatment of barley prior to malting followed by *Lact. plantarum* E76 addition into the steeping waters was found to be an effective combination in controlling *Fusarium* flora during malting (Olkku *et al.* 2000).

The transfer of knowledge obtained from laboratory experiments with pure cultures and defined conditions into the real complex malting process is a challenging area which definitely needs further study. Furthermore, optimal control of microflora proliferation during the malting process could be attained by combining several suitable treatments. Our further work has been directed towards the combination of protective cultures with other microflora management techniques. The results of the present study indicate that the use of LAB offers a potential alternative as a natural, food-grade biocontrol agent. Strains with known and selected characteristics could be used for management of problems caused by *Fusarium* fungi and for improving the safety of cereal-based products.

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