

New types of antimicrobial compounds produced by *Lactobacillus plantarum*

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M.-L. NIKU-PAAVOLA, A. LAITILA, T. MATTILA-SANDHOLM AND A. HAIKARA. 1999. New types of antimicrobial compounds were identified in the culture filtrate of *Lactobacillus plantarum* VTT E-78076. Activity was detected in the low molecular mass fraction separated by gel chromatography. This fraction totally inhibited the growth of the Gram-negative test organism, *Pantoea agglomerans* (*Enterobacter agglomerans*) VTT E-90396. Characteristic compounds from this fraction were identified by GC/MS-analysis and the identification was confirmed using pure commercial reference compounds in identical chromatographs and in antimicrobial tests. The active fraction included benzoic acid (CAS 65-85-0), 5-methyl-2,4-imidazolidinedione (CAS 616-03-5, methylhydantoin), tetrahydro-4-hydroxy-4-methyl-2H-pyran-2-one (CAS 674-26-0, mevalonolactone) and 3-(2-methylpropyl)-2,5-piperazinedione (CAS 5845-67-0, cyclo(glycyl-L-leucyl)). These compounds in concentrations of 10 ppm inhibited growth of the test organism by 10–15% when acting separately, but 100% when all were applied together with 1% lactic acid. The inhibition was 40% by 1% lactic acid alone. The compounds were also active against *Fusarium avenaceum* (*Gibberella avenacea*) VTT D-80147. The inhibition was 10–15% by separate compounds in concentrations of 10 ppm and maximally 20% in combinations. Fungal growth was not inhibited by lactic acid. Inhibition by unfractionated *Lact. plantarum* culture filtrate was 37% and by the low molecular mass fraction, 27%.

INTRODUCTION

Fermentation with lactic acid bacteria (LAB) has long been used in the processing of different foods. Milk, meat and vegetable products, as well as silage, have been prepared using LAB starters in order to improve the flavour and texture of the product. Lactic acid bacteria have been shown to enhance the stability and nutritional value of food products by preventing the growth of pathogenic and spoilage microbes. In addition to these conventional fermentation processes, LAB have recently been found to be beneficial in new applications, e.g. in malting (Haikara *et al.* 1993; Haikara and Laitila 1995) and in wine fermentation (Buckenhueskes 1993). Acidified wort for alcohol-free beer is produced using immobilized LAB (Pittner and Back 1995). Interest in the metabolism of LAB has increased in recent years because natural pres-

ervation methods are required for minimally processed foods and functional food products, which are becoming increasingly popular.

Lactic acid bacteria compete with other microbes by secreting antagonistic compounds and modifying the micro-environment by their metabolism (Lindgren and Dobrogosz 1990). Several active compounds, and their modes of action, have been characterized (de Vuyst and Vandamme 1994). The antimicrobial metabolites can be divided into low molecular mass compounds (below 1000) and bacteriocins (molecular mass over 1000). The effect of all these compounds is due to their specific action on the surrounding microflora (Daeschel 1989; Lindgren and Dobrogosz 1990).

Bacteriocins are a heterogeneous group of antibacterial peptides and proteins that vary in their spectrum of activity, mode of action, molecular mass, genetic origin and biochemical properties (de Vuyst and Vandamme 1994; Abee *et al.* 1995). They have an inhibitory effect only on closely related species and on other Gram-positive organisms.

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The biologically active, non-proteinaceous low molecular mass compounds produced by LAB are poorly characterized, although the existence of such compounds has frequently been reported (e.g. Lonvaud-Funel and Joyeux 1993; de Vuyst and Vandamme 1994; Gourama and Bullerman 1995). These substances differ from bacteriocins in their wide spectrum of activity against both Gram-positive and Gram-negative bacteria and fungi.

Studies at VTT (Technical Research Centre of Finland) Biotechnology and Food Research revealed a group of LAB which have antagonistic activities against Gram-negative bacteria and *Fusarium* fungi (Haikara *et al.* 1993; Haikara and Laitila 1995). The preliminary characterization of *Lactobacillus plantarum* and *Pediococcus pentosaceus* starter cultures revealed new types of antimicrobial substances with low molecular mass and features not previously reported for LAB microbicides (Haikara and Niku-Paavola 1993). The aim of this study was to characterize and identify the low molecular mass antimicrobial compounds produced by *Lact. plantarum* VTT E-78076.

MATERIALS AND METHODS

Micro-organisms

All the test organisms were obtained from the VTT Culture Collection. *Lactobacillus plantarum* VTT E-78076 (E 76) was originally isolated from beer. *Pantoea agglomerans* (basonyms *Enterobacter agglomerans* and *Herminia herbicola*) VTT E-90396 (E 396) and *Fusarium avenaceum* (teleomorph *Gibberella avenacea*) VTT D-80147 (D 147) originated from barley kernels.

Preparation of the culture filtrate

Lactobacillus plantarum was cultivated in MRS broth (Oxoid) at 30 °C for 72 h. The culture was centrifuged at 3000 *g* for 10 min and the supernatant fluid was filtered through a 45 µm pore-size filter (Millex-HA, Millipore, S.A., Molsheim, France). The sterile filtrate was stored at +4 °C.

Effect of heat and enzymes on the antimicrobial activity of the culture filtrate

The culture filtrate containing antimicrobial activity was exposed to heat treatment either in a water-bath at 80 °C for 1 h or in an autoclave at 120 °C for 15 min. Sensitivity to proteolytic enzymes was tested with four commercial proteolytic enzyme preparations (trypsin, Merck; α-chymotrypsin, Merck; pronase E, Merck; and protease XIII, Sigma). Culture filtrate (4.5 ml) and enzyme (0.5 ml, 1.5 or 10 mg ml⁻¹ in 0.1 mol l⁻¹ sodium phosphate buffer, pH 7.4) were incubated in a water-bath at 37 °C for 1 h. In order to exclude

the possible effect of H₂O₂ formation, the antimicrobial activity of the culture filtrate was also assessed in the presence of catalase (1 mg ml⁻¹, Sigma). Antimicrobial activities of the extracts after different treatments were determined by an agar diffusion method using a disc test. The Gram-negative bacterium, *P. agglomerans* E 396, grown in Nutrient broth (Difco) at 30 °C for 24 h, was used as an indicator for antimicrobial activity. Melted agar (15 ml) was mixed with 300 µl of a 24 h culture broth, diluted 10⁻², in a sterile Petri dish. When the agar had hardened, antibiotic test discs (diameter 12.7 mm, Schleicher & Schull, Dassel, Germany) were placed on the agar surface and 100 µl of the sample were spotted onto the disc. After incubation at 30 °C for 24 h, the diameter of the clear inhibition zone around the disc was measured.

Separation of the low molecular mass antimicrobial fraction

The culture filtrate of *Lact. plantarum* and, as a reference, MRS-broth without inoculum, were fractionated by gel chromatography. The fractionation was carried out in a Sephadex G-10 (Pharmacia, Uppsala, Sweden) column (360 ml) with distilled water. The elution rate was 0.5 ml min⁻¹, and fractions of 5 ml were collected. The fractions were monitored at 280 nm and analysed for carboxylic acids by HPLC, and for antimicrobial activity by turbidometry. The fractions of *Lact. plantarum* culture filtrate containing antimicrobial activity, the adjacent inactive fractions and the corresponding fractions from MRS-broth without inoculum, were compared by GC/MS.

Analysis of carboxylic acids

Acids were determined by HPLC with a u.v.-detector (Waters Lambda-Max Model 481 at 210 nm) and an Aminex HPX-87H cation exchanger, H form (300 × 7.8). The acids were eluted with 3 mmol l⁻¹ sulphuric acid at 65 °C.

Gas chromatography-mass spectrometry

Fractions from gel chromatography were analysed using GC/MS. A Varian 3400 gas chromatograph equipped with a CTC 2000 autosampler was interfaced to an IncoS-50 mass-selective detector. The capillary column was a 50 m non-polar HP-5 (Hewlett Packard, i.d. 0.2 mm, film thickness 0.33 µm). The gas chromatograph oven temperature was held at 80 °C for 1 min, then increased to 280 °C at 5 °C min⁻¹ and held at 320 °C for a further 5 min. Helium was used as carrier gas with a velocity of 30 cm s⁻¹ measured at 80 °C. The splitless time was 0.25 min and the injection volume was 1 µl. Compounds characteristic only for the antimicrobial fractions of *Lact. plantarum* were identified. The corresponding fractions of non-inoculated MRS broth and the

inactive fractions of *Lact. plantarum* were used as negative controls. In the identification, the mass spectra library (Wiley Registry of Mass Spectral Data with Structures, 6th edn, Palisade Corp., USA) was used. The identification was based on 90% similarity between the spectra of unknown and reference.

Confirmation of the identity of the active compounds

The identified compounds, when commercially available, were tested for their antimicrobial activity. The active reference compounds found were further tested by gel filtration chromatography and a GC-MS method to compare their retention times and spectra with those of the active fraction from *Lact. plantarum*.

Testing for antimicrobial activity of the low molecular mass antimicrobial compounds

Culture media and growth conditions for indicator organisms prior to testing. *Pantoea agglomerans* E 396 was grown in Nutrient Broth (Difco) at 30 °C for 24 h. This inoculum provided $1-3 \times 10^8$ cfu ml⁻¹ determined on Plate Count agar (Difco). The suspension was diluted 10^{-2} and the inoculum used was calibrated to 10^4 cfu per well. The fungus *F. avenaceum* was induced to sporulate on 1% CMC broth (10 g l⁻¹ carboxymethylcellulose, 1 g l⁻¹ NH₄NO₃, 1 g l⁻¹ KH₂PO₄, 0.5 g l⁻¹ MgSO₄·7H₂O, 1 g l⁻¹ yeast extract) cultivated with shaking (120 rev min⁻¹) at 25 °C for 7 d (Booth 1977). The suspension was diluted 10^{-1} with 0.005% Tween-80 v/v. Glass beads (10–15 per test-tube) were used to break the mycelium and the mycelial debris was removed by filtration through glass wool. The number of spores was measured in a Thoma counting chamber. The spore suspension was adjusted to 1000 spores per well.

Testing for antimicrobial activity with Bioscreen. An automated turbidometer, Bioscreen® (Labsystems Oy, Helsinki, Finland), was used for measuring the microbicidal potential of the antimicrobial compounds (Skyttä and Mattila-Sandholm 1991; Skyttä *et al.* 1993). The pH of the samples was adjusted to 4.0 with NaOH. A 30 µl sample of the suspension to be studied and 30 µl of the test organism in growth medium were dispensed to microtitre plate wells with 240 µl of the growth medium. In the control sample wells, the antimicrobial agent was replaced by an equal volume of sterile growth medium. *Fusarium avenaceum* D 147 was incubated with shaking at 25 °C for 72 h and *P. agglomerans* E 396, with shaking at 30 °C for 24 h. All determinations were carried out with three or 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 microbial growth, and

area-reduction percentage values were used to describe the inhibitory effects of the antimicrobial compounds.

RESULTS

Effect of heat treatment and enzymes on antimicrobial activity

It had previously been observed that the antimicrobial activity of starter cultures was strongly dependent on the cultivation medium (unpublished results). The production was maximal in MRS medium, which was therefore used in this study. The effects of heat treatments and enzymes on the antimicrobial activity of the culture filtrate of *Lact. plantarum* were measured prior to the purification steps. Culture filtrate gave a clear inhibition zone with a diameter of 18 mm. MRS medium used as a control sample did not inhibit the growth of *P. agglomerans*. Heat treatments did not reduce the antimicrobial activity of culture filtrates. The activity could still be demonstrated even after heating at 120 °C for 15 min in the autoclave. The antimicrobial activity could not be inactivated with proteolytic enzymes or catalase. This suggested that inhibition of the indicator strain could not have resulted from hydrogen peroxide, and that the inhibitory compounds were not proteinaceous in nature. Moreover, after gel chromatography, inhibitory activity was found in the low molecular mass fraction eluted after lactic acid (Fig. 1).

Separation and identification of compounds from the antimicrobial fraction

The antimicrobial compounds were separated from the culture filtrate of *Lact. plantarum* using a Sephadex G-10 column, which fractionates compounds having a molecular mass lower than 700 Da. The antimicrobial activity of the fractions was estimated using *P. agglomerans* as the test organism. A typical elution pattern is presented in Fig. 1. Lactic acid, which is often considered as the main antimicrobial compound of LAB, was clearly separate from the antimicrobial fraction. Growth inhibition of *P. agglomerans* was 100% by the unfractionated culture filtrate and the antimicrobial low molecular mass fraction, LMM (Table 1). The fractions from gel chromatography, containing lactic acid up to 7 g l⁻¹, were not inhibitory, although inhibition by 1% lactic acid was 40%. Growth of the other test organism, *F. avenaceum*, was 37% inhibited by the culture filtrate, whereas inhibition by the separated antimicrobial fraction was, surprisingly, only 27% (Fig. 2). It appeared that the antifungal activity included the LMM fraction but obviously, other compounds eliminated by the fractionation as well. The potential candidates are the higher molecular mass compounds not tested against *F. avenaceum*.

In order to provide a negative control, fractionation was

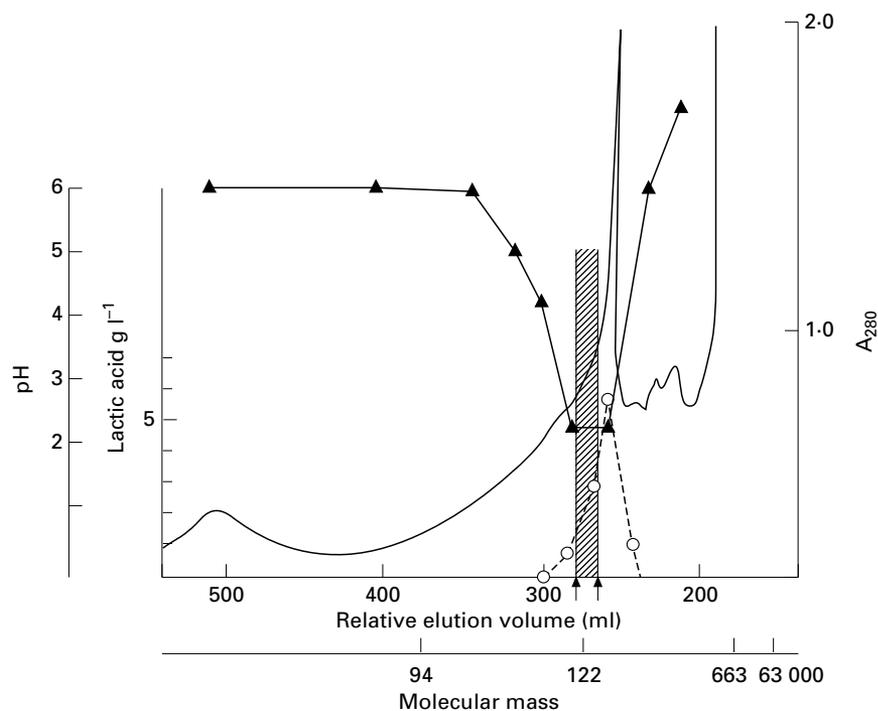


Fig. 1 Fractionation of antimicrobial activity by gel chromatography on Sephadex G 10; elution with water. Sample: culture filtrate of *Lactobacillus plantarum* grown in MRS broth for 72 h. Elution volumes of molecular mass standards (phenol 94, benzoic acid 122, NAD 663, bovine serum albumin 63 000 Da) are indicated by vertical lines. (—) A₂₈₀; (▲) pH; (○) concentration of lactic acid; (▨), antimicrobial activity

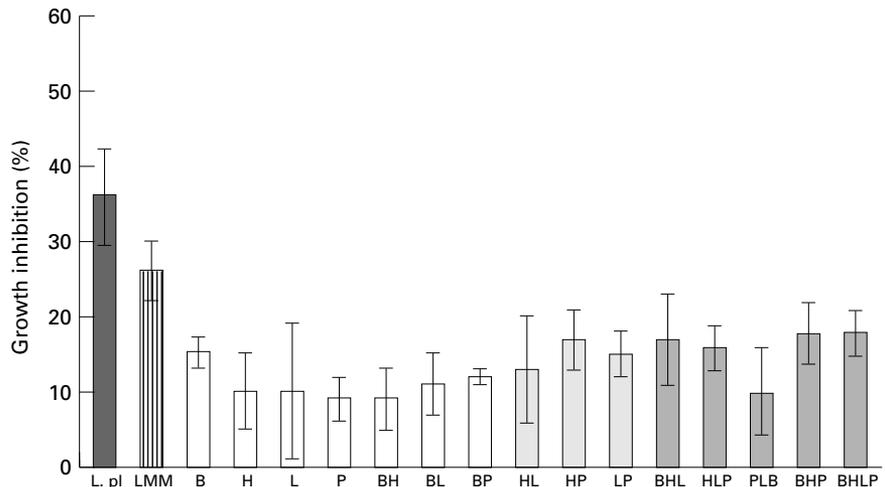
Sample	Growth inhibition %
<i>Lactobacillus plantarum</i> culture filtrate	100
LMM	100
Benzoic acid 10 ppm	10
Methylhydantoin 10 ppm	10
Mevalonolactone 10 ppm	15
Methylhydantoin 10 ppm + benzoic acid 10 ppm	15
Mevalonolactone 10 ppm + benzoic acid 10 ppm	15
Methylhydantoin 10 ppm + mevalonolactone 10 ppm	10
Mevalonolactone 10 ppm + methylhydantoin 10 ppm + benzoic acid 10 ppm	15
Lactic acid 1%	40
Benzoic acid 10 ppm + lactic acid 1%	5
Methylhydantoin 10 ppm + lactic acid 1%	30
Mevalonolactone 10 ppm + lactic acid 1%	60
Mevalonolactone 10 ppm + methylhydantoin 10 ppm + lactic acid 1%	30
Mevalonolactone 10 ppm + benzoic acid 10 ppm + lactic acid 1%	30
Methylhydantoin 10 ppm + benzoic acid 10 ppm + lactic acid 1%	15
Mevalonolactone 10 ppm + methylhydantoin 10 ppm + benzoic acid 10 ppm + lactic acid 1%	100

Table 1 Growth inhibition of *Pantoea agglomerans* by antimicrobial compounds

also performed with non-inoculated MRS broth. This elution pattern (not shown) was clearly different from that of the culture filtrate of *Lact. plantarum*. The amount of compounds

with molecular masses higher than 700 Da was considerably reduced, and the low molecular mass compounds mainly disappeared during growth of *Lact. plantarum*. No peaks were

Fig. 2 Growth of *Fusarium avenaceum* in the presence of antimicrobials; tested by turbidometry. *Lactobacillus plantarum* unfractionated culture filtrate, L.pl; low molecular mass fraction, LMM; and pure reference compounds at a concentration of 10 ppm each (B, benzoic acid, H, methylhydantoin, L, mevalonolactone, P, cyclo(L-leucylglycine). Values are means of three–seven replicates from two different Bioscreen tests. Error bars represent standard deviations



obtained from the non-inoculated MRS broth at the elution volume of lactic acid or antimicrobial compounds. The pH in the non-inoculated fractions remained constant at 7–7.5, whereas the pH in the culture filtrate fractions decreased to pH 2 in fractions containing lactic acid or antimicrobial compounds. It appeared that fractionation in Sephadex G-10 was only partly dependent on molecular mass and that it was also due to adsorption of the antimicrobial compounds on the gel matrix. Thus, elution volumes cannot be used to predict the molecular masses of the compounds in the antimicrobial fraction.

The antimicrobial fraction and the inactive fractions were analysed by GC/MS. All the compounds present in the active fraction are shown in Fig. 3. The corresponding fraction from MRS broth did not contain similar peaks but only a few with very low intensity and different scan numbers. The inactive low molecular mass fractions from *Lact. plantarum* did, however, contain the same major peaks as the active fraction. The peaks characteristic only for the antimicrobial fraction of *Lact. plantarum* were those with low intensity in Fig. 3. They were identified by comparing their spectra with those in the mass spectra library. Identifications in which an identity of 90% with the reference spectrum was obtained were considered reliable.

The identified compounds specific for the antimicrobial fraction of *Lact. plantarum* included aromatic and heterocyclic compounds. Their concentrations were mainly very low, only 1–10 ppm as estimated from the peak intensity.

Confirmation of the identity of the active compounds

Only those identified compounds which were commercially available could be tested as pure reference compounds for growth inhibiting activity towards *P. agglomerans* and *F.*

avenaceum in the turbidometric measurements. The doses in assays were adjusted to correspond to the true concentrations of the identified compounds in the *Lact. plantarum* fraction, 10 ppm. The activities were compared with those of the unfractionated culture filtrate of *Lact. plantarum* and of the low molecular mass fraction after gel chromatography. The compounds most clearly implicated in the antimicrobial effects of *Lact. plantarum* were benzoic acid (CAS 65–85–0), mevalonolactone (CAS 674–26–0), methylhydantoin (CAS 616–03–5) and cyclo(glycyl-L-leucyl) (CAS 5845–67–0) (Fig. 3). The retentions of these reference compounds were confirmed to be similar to those of the compounds in the antimicrobial fraction of *Lact. plantarum* in gel chromatography and in the GC-MS method. The identity between the spectra was confirmed by GC-MS analysis.

Growth of *P. agglomerans* was totally inhibited by the unfractionated culture filtrate of *Lact. plantarum* as well as by the low molecular mass fraction. Inhibition by the individual pure reference compounds was 10–15% and by lactic acid, 40% (Table 1). Mevalonolactone and lactic acid together inhibited growth by 60%, indicating a slight synergy. The other combinations were less effective than the summarized individual effects. It appeared that methylhydantoin and benzoic acid were the compounds which decreased the expected growth inhibiting activity of the combinations. The fact that the combination of all reference compounds and lactic acid had an inhibition effect as strong as that of the unfractionated culture filtrate and the low molecular mass fraction was exceptional.

The antimicrobial activities of the identified compounds were also tested against *F. avenaceum* (Fig. 2). Growth inhibition was 37% by the culture filtrate of *Lact. plantarum* and 27% by the low molecular mass fraction. The individual reference compounds at a concentration of 10 ppm caused 10–

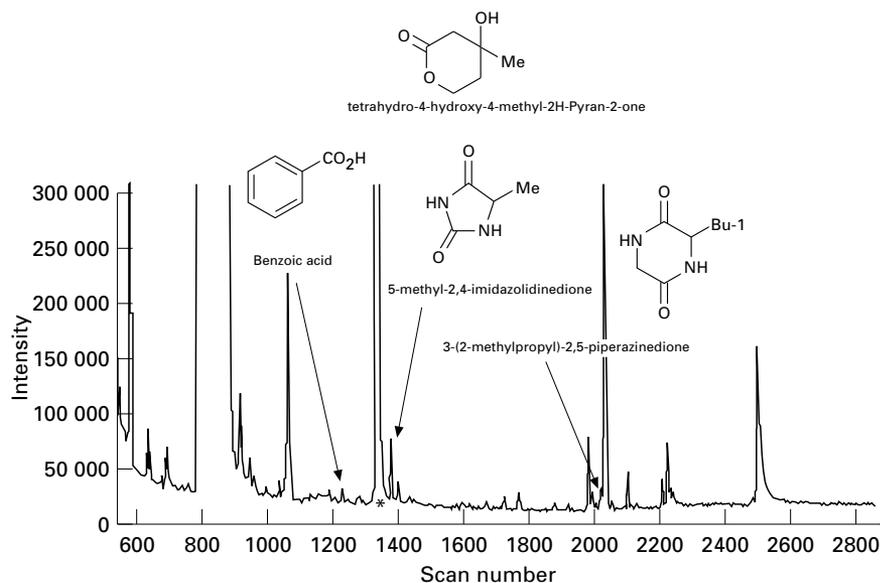


Fig. 3 Characteristic compounds of the antimicrobial fraction of *Lactobacillus* culture filtrate, analysed by GC/MS. Arrows indicate compounds shown to have antimicrobial activity. * Retention of mevalonolactone

15% inhibition. Combinations of the reference compounds increased the inhibition maximally to 20%. In accordance with the results obtained with *P. agglomerans*, the presence of methylhydantoin and benzoic acid in the combinations decreased the expected inhibitory effect. Lactic acid did not affect the growth of *F. avenaceum* either alone or in combination.

DISCUSSION

The antimicrobial compounds produced by LAB are natural preservatives as such and could be used as preparations for increasing the shelf-life and safety of minimally processed foods. Hitherto, nisin is the only bacteriocin which has been accepted by the World Health Organization as a preservative in the food industry (Vandenbergh 1993). The range of applications of antimicrobial metabolites of LAB will certainly grow in future because of their wide spectrum of activity. Lactic acid bacteria antimicrobials can be exploited in feed applications and furthermore, in non-food applications such as pharmaceuticals. However, a broader spectrum is needed than that of bacteriocins, which mainly inhibit Gram-positive bacteria. Low molecular mass compounds produced by LAB are active against both Gram-positive and Gram-negative bacteria and moulds. They have not yet been effectively exploited in commercial applications because of their inadequate structural characterization. Reuterin, a mixed product of *L. reuteri* containing β -hydroxypropionaldehyde, its hydrated acetal and the cyclic dimer of the aldehyde, has long been the only structurally characterized low molecular mass antimicrobial preparation (Talarico and Dobrogosz 1989).

In this study, new types of antimicrobial compounds pro-

duced by *Lact. plantarum* VTT E-78076 were identified. They were all low molecular mass compounds. The difficulties in purification and identification of this type of compound were clearly demonstrated. The main difficulty was that several compounds were involved in the co-operative action and that the concentrations of the compounds were extremely low.

The identified low molecular mass compounds of *Lact. plantarum* inhibited the growth of Gram-negative *P. agglomerans* totally when all acting in co-operation with lactic acid. They also showed activity against the fungus *F. avenaceum*. The growth inhibition effect of the reference compounds, separately and in combinations, towards *F. avenaceum* was lower than that of the low molecular mass antimicrobial fraction. Furthermore, the activity of the low molecular mass fraction was less than that of unfractionated culture filtrate. It appears that not all the antimicrobial compounds of *Lact. plantarum* were detected here, and also that the *Lact. plantarum* compounds inhibiting *P. agglomerans* may not necessarily be the same as those effective towards *F. avenaceum*.

The results showed that mevalonolactone, identified in the antimicrobial fraction of *Lact. plantarum*, inhibited growth of *P. agglomerans* in synergy with lactic acid, while other identified compounds, methylhydantoin and benzoic acid, decreased the summarized individual effects in combination. Thus, the 100% inhibition by the combination of all reference compounds and lactic acid was surprising. It might have been due to the fact that the antimicrobial compounds in mixtures interacted with each other as well as with the test organisms. Depending on the compounds present, the reactions may proceed differently, resulting in either synergistic or antagonistic action. In the combination resulting in 100% inhi-

bition, the synergistic modifications may have been stronger than the antagonistic ones. The mechanism of the inhibition is unknown and needs to be studied further.

The compounds identified were chemically quite different from one another. Their common features were small size and aromatic or heterocyclic structure. Another cyclic compound, pyroglutamic acid, produced by a *Lact. casei* subsp., has also been introduced as an antimicrobial agent (Huttunen *et al.* 1995). The inhibitory effects of pyroglutamic acid, reuterin and the compounds identified in this work are difficult to compare because different biological tests were applied in each case and the activities were differently expressed. More research is needed to characterize the activities of lactobacilli precisely.

The antimicrobial compounds identified here have been studied previously because of other interesting biological properties. Benzoic acid is one of the oldest chemical preservatives used in the cosmetic, drug and food industries. Benzoic acid has GRAS (Generally Recognized As Safe) status and sodium benzoate was the first chemical preservative approved by the US Food and Drug Administration for use in foods (Jay 1992). The activity of benzoic acid (pKa 4.19) is greatest at low pH values and the use of this acid as a food preservative has been limited to those products which are acid in nature, such as fruit products, tomato ketchup and soft drinks. Most yeasts and fungi are inhibited by 0.05–0.1% of the undissociated acid (Chipley 1993), although it is usually applied in far greater concentrations. The action here in rather low concentration in synergy with other antimicrobial agents is a new feature. Methylhydantoin, the peptide cyclo-(glycyl-L-leucyl) and mevalonolactone have been used, e.g. in the synthesis and preparation of pharmaceuticals.

All the compounds introduced in this work have several interesting potential applications in the food, feed and pharmaceutical industries. Further screening of LAB antimicrobials may reveal other novel applications.

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