ANTIMICROBIAL COMPOUNDS AND EXTRACELLULAR POLYSACCHARIDES PRODUCED BY LACTIC ACID BACTERIA: STRUCTURES AND PROPERTIES

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Academic Dissertation

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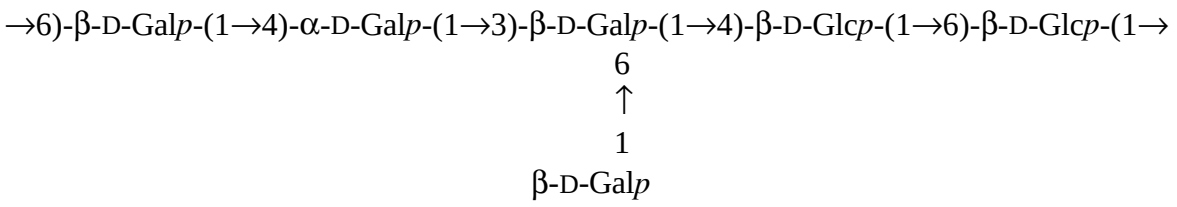
ABSTRACT

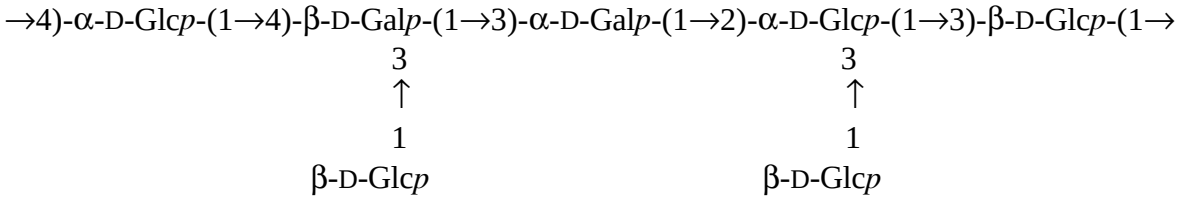
Antimicrobial compounds and exopolysaccharides (EPSs) produced by dairy lactic acid bacteria (LAB) were studied. These compounds were separated and purified, and their structures were investigated. The activity of the antimicrobial compounds and the rheological properties of the EPSs were also studied.

Thirteen *Lactobacillus* and five *Pediococcus* strains were shown to produce a low-molecular-mass antimicrobial compound, which was separated and purified by chromatographic methods. Identification by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry showed that the antimicrobial compound was 2-pyrrolidone-5-carboxylic acid (PCA), also known as pyroglutamic acid. The technique of anion exchange chromatography developed in this study was essential for effective separation of PCA from lactic acid, facilitating the identification of PCA.

PCA has a wide spectrum of antimicrobial activity against various food-borne spoilage bacteria, including the genera *Bacillus*, *Enterobacter*, *Escherichia*, *Klebsiella*, and *Pseudomonas*. The activity of PCA was stable after heat treatments. Compared to lactic acid, PCA had slightly lower antimicrobial activity. On the basis of the results of this study, PCA can be considered, following the identification of reuterin produced by *Lb. reuteri*, to be another well identified low-molecular-mass antimicrobial compound produced by LAB with a wide spectrum of activity.

Lactic acid bacteria are known to produce viscous or non-viscous EPSs. In this study, ten LAB strains have been found to produce EPSs, which have been isolated. Studies on the EPSs produced by *Lb. helveticus* strains showed that strain Äki4 produced a neutral EPS which was found to be not viscous, whereas strain Lb161 produced a neutral and viscous EPS. Sugar analysis, methylation analysis, and one- and two-dimensional NMR spectroscopy showed that the EPSs produced by *Lb. helveticus* Äki4 and Lb161 consisted of a hexasaccharide and a heptasaccharide repeating unit, respectively:





Further studies on the EPSs produced by Finnish fermented milk ‘viili’ strains showed that the slime-forming *Lactococcus lactis* ssp. *cremoris* strains (ARH53, ARH74, ARH84, ARH87, B30) produced EPSs of relatively high viscosities. These EPSs differed in viscosity in aqueous solutions, and the viscosity was temperature, pH and salt dependent. Addition of EPS to skim milk significantly increased the viscosity, and gelation occurred at 40 °C. ^{1}H NMR spectroscopy showed that the EPSs had a similar or probably identical structure to the one previously reported.

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LIST OF PUBLICATIONS

This thesis is based on the following publications which are referred to in the text by Roman numerals. Additional data are also presented.

I Huttunen, E., Noro, K., and **Yang, Z.** 1995. Purification and identification of antimicrobial substances produced by two *Lactobacillus casei* strains. Int. Dairy J. 5, 503-513.

II **Yang, Z.**, Suomalainen, T., Mäyrä-Mäkinen, A., and Huttunen, E. 1997. Antimicrobial activity of 2-pyrrolidone-5-carboxylic acid produced by lactic acid bacteria. J. Food Prot. 60, 786-790.

III Staaf, M., Widmalm, G., **Yang, Z.**, and Huttunen, E. 1996. Structural elucidation of an extracellular polysaccharide produced by *Lactobacillus helveticus*. Carbohydr. Res. 291, 155-164.

IV Staaf, M., **Yang, Z.**, Huttunen, E., and Widmalm, G. 2000. Structural elucidation of the viscous exopolysaccharide produced by *Lactobacillus helveticus* Lb161. Carbohydr. Res. (in press).

V **Yang, Z.**, Huttunen, E., Staaf, M., Widmalm, G., and Tenhu, H. 1999. Separation, purification and characterization of extracellular polysaccharides produced by slime-forming *Lactococcus lactis* ssp. *cremoris* strains. Int. Dairy J. 9, 631-638.

ABBREVIATIONS

Ac	acetyl
BMM	basal minimum medium
CDM	chemically defined medium
CM	carboxymethyl
COSY	correlated spectroscopy
CFU	colony forming units
DEAE	diethylaminoethyl
DEPT	distortionless enhancement by polarization transfer
DNA	deoxyribonucleic acid
D_2O	deuterium oxide
EPS	exopolysaccharide
EI	electron impact
FAB	fast atom bombardment
Fru	fructose
Fuc	fucose
Gal	galactose
GalNAc	*N*-acetylgalactosamine
GC	gas chromatography
gHMBC	gradient selected heteronuclear multiple-bond correlation
gHSQC	gradient selected heteronuclear single quantum coherence
GLC	gas-liquid chromatography
Glc	glucose
GlcNAc	*N*-acetylglucosamine
GRAS	Generally Recognized As Safe
G3P	*sn*-glycerol-3-phosphate
HMBC	heteronuclear multiple-bond correlation
HMM	high-molecular-mass
HOHAHA	homonuclear Hartmann-Hahn spectroscopy
HPLC	high performance liquid chromatography
KCA	calcium citrate agar (Valio)
KKNO	Valio's Culture Collection, Valio Ltd, Helsinki, Finland
LAB	lactic acid bacteria
Lb.	*Lactobacillus*
Lc.	*Lactococcus*
Leuc.	*Leuconostoc*
LMM	low-molecular-mass
Man	mannose
MIC	minimum inhibitory concentration
MRS	Man-Rogosa-Sharpe
MS	mass spectrometry
NADH	nicotinamide adenine hydroxy dinucleotide
NCFB	National Collection of Food Bacteria, Reading, U.K.
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
P.	*Pediococcus*

PCA	2-pyrrolidone-5-carboxylic acid
ppm	parts per million
Rha	rhamnose
RP-HPLC	reversed-phase high-performance liquid chromatography
S.	*Streptococcus*
SDM	semi-defined medium
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TOCSY	total correlation spectroscopy
UV	ultraviolet
w/v	weight per volume
1D	one-dimensional
2D	two-dimensional

1. INTRODUCTION

In a variety of ecological niches, microorganisms compete with each other for survival and through evolution form unique flora. In some food ecosystems, lactic acid bacteria (LAB) constitute the dominant microflora. These organisms are able to produce antimicrobial compounds against competing flora, including food-borne spoilage and pathogenic bacteria (Daeschel 1989, Davidson and Hoover 1993). Under unfavorable environmental conditions many species of LAB also produce exopolysaccharides (EPSs), which protect themselves against desiccation, bacteriophage and protozoan attack (Whitfield 1988, Roberts 1995, Weiner *et al*. 1995).

Lactic acid bacteria provide the major preservative effects in food fermentation which mankind has practiced for thousands of years. The primary antimicrobial effect exerted by LAB is the production of lactic acid and reduction of pH (Daeschel 1989). In addition, LAB produce various antimicrobial compounds, which can be classified as low-molecular-mass (LMM) compounds such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione), uncharacterized compounds, and high-molecular-mass (HMM) compounds like bacteriocins (Piard and Desmazeaud 1991, 1992, Ouwehand 1998). Among bacteriocins so far characterized, nisin is best defined, and the only purified bacteriocin produced by LAB that has been approved for use in food products (Hansen 1994). A LMM antimicrobial compound, reuterin, has also been chemically identified (Talarico and Dobrogosz 1989), and the reuterin-producing *Lactobacillus reuteri* strain has been applied as a probiotic in dairy products (Rothschild 1995).

The EPSs produced by LAB are either present as a capsule attached to the cell surface, or secreted into the environment (Cerning 1990). Based on their sugar compositions, the EPSs can be divided into homopolysaccharides, composed of a single type of monosaccharide, and heteropolysaccharides, containing several types of monosaccharide (Gruter 1992). Dextran is the most important homopolysaccharide, which is a glucan produced, e.g. by *Leuconostoc mesenteroides* (Franz 1986). The heteropolysaccharides produced by LAB are generally composed of repeating units of up to eight monosaccharide residues; the chain length and degree of branching vary with the producing strains. The rheological properties of the polysaccharides depend on the monomeric composition, the number of side chains, the chain length and the charge (neutral or anionic) of the polysaccharides, as well as the anomeric configuration of the monosaccharides and the sequence in which they are arranged. The EPSs produced by LAB may act as viscosifying agents to improve the texture and consistency of fermented foods (Cerning 1990, Sikkema and Oba 1998). Since LAB are food-grade microorganisms with the GRAS status (Generally Recognized As Safe), the use of the secreted EPSs as natural alternatives to produce all-natural food products without additives has received increased attention. It has also been claimed that EPSs isolated from LAB cultures have antitumor activity (Oda *et al*. 1983).

Lactic acid bacteria are able to produce a large variety of compounds which contribute to the flavor, color, texture and consistency of fermented foods. However, the present study focuses on two potentially important group of compounds, antimicrobial compounds and EPSs, which differ largely in their chemistry and functionalities. Attention has been paid to developing methods suitable for separation and purification of LMM antimicrobial compounds aiming at inhibition of food-borne spoilage bacteria. In order to understand the relation between the structures and rheological properties of the EPSs, a knowledge of their primary molecular structures is required. In this study, the primary molecular structures of the EPSs produced by *Lb. helveticus* strains have been studied by NMR spectroscopy. The EPSs produced by several slime-forming *Lactococcus lactis* ssp. *cremoris* strains have been characterized to understand the roles of the EPSs in the rheology of fermented foods.

2. LITERATURE REVIEW

2.1. Antimicrobial compounds produced by lactic acid bacteria

Lactic acid bacteria are a physiologically diverse group of organisms, which can be generally described as Gram-positive, nonsporing cocci or rods with lactic acid as the major product of carbohydrate fermentation. Traditionally, LAB comprise four genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. However, several new genera have been suggested for inclusion in the group of LAB due to a recent taxonomic revision (Axelsson 1998). The genus *Streptococcus* has been reorganized into *Enterococcus*, *Lactococcus*, *Streptococcus* and *Vagococcus*.

LAB are involved in the fermentation of a range of milk, meat, cereal and vegetable foods (McKay and Baldwin 1990). The antimicrobial compounds produced by LAB can inhibit the growth of pathogenic bacteria of possible contaminants in the fermented products (Raccah *et al*. 1979, Smith and Palumbo 1983, Cintas *et al*. 1998). In the past two decades, there have been many reports on the bacteriocins produced by LAB. These bacteriocins are of a proteinaceous nature and they have been grouped into class I, lantibiotics which are small peptides (e.g. nisin), class II, small heat-stable peptides, class III, large heat-labile proteins, and class IV, complex bacteriocins which are not well defined (Klaenhammer 1993). In the following text, the LMM antimicrobial compounds produced by LAB will be discussed.

2.1.1. Organic acids

Fermentation by LAB is characterized by the accumulation of organic acids and the accompanying reduction in pH. The levels and types of organic acids produced during the fermentation process depend on the species of organisms, culture composition and growth conditions (Lindgren and Dobrogosz 1990). The antimicrobial effect of organic acids lies in the reduction of pH, as well as the undissociated form of the molecules (Gould 1991, Podolak *et al*. 1996). It has been proposed that the low external pH causes acidification of the cell cytoplasm, while the undissociated acid, being lipophilic, can diffuse passively across the membrane (Kashket 1987). The undissociated acid acts by collapsing the electrochemical proton gradient, or by altering the cell membrane permeability which results in disruption of substrate transport systems (Smulders *et al*. 1986, Earnshaw 1992).

Lactic acid is the major metabolite of LAB fermentation where it is in equilibrium with its undissociated and dissociated forms, and the extent of the dissociation depends on pH. At low pH, a large amount of lactic acid is in the undissociated form, and it is toxic to many bacteria, fungi and yeasts. However, different microorganisms vary considerably in their sensitivity to lactic acid. At pH 5.0 lactic acid was inhibitory toward spore-forming bacteria but was ineffective against yeasts and moulds (Woolford 1975). It was possible to grow *Aspergillus parasiticus* NRRL 2999 in a medium containing 0.5 or 0.75% lactic acid at pH 3.5 or 4.5 (El-Gazzar *et al*. 1987). Lindgren and Dobrogosz (1990) showed that at different pH ranges the minimum inhibitory concentration (MIC) of the undissociated lactic acid was different against *Clostridium tyrobutyricum*, *Enterobacter* sp. and *Propionibacterium freudenreichii* ssp. *shermanii*. In addition, the stereoisomers of lactic acid also differ in antimicrobial activity, L-lactic acid being more inhibitory than the D-isomer (Benthin and Villadsen 1995).

Acetic and propionic acids produced by LAB strains through heterofermentative pathways, may interact with cell membranes, and cause intracellular acidification and protein denaturation (Huang *et al*. 1986). They are more antimicrobially effective than lactic acid due to their higher pKa values (lactic acid 3.08, acetic acid 4.75, and propionic acid 4.87), and higher percent of undissociated acids than lactic acid at a given pH (Earnshaw 1992). Acetic acid was more inhibitory than lactic and

citric acids toward *Listeria monocytogenes* (Ahamad and Marth 1989, Richards *et al*. 1995), and toward the growth and germination of *Bacillus cereus* (Wong and Chen 1988). Acetic acid also acted synergistically with lactic acid; lactic acid decreases the pH of the medium, thereby increasing the toxicity of acetic acid (Adams and Hall 1988).

2.1.2. Hydrogen peroxide and carbon dioxide

Hydrogen peroxide is produced by LAB in the presence of oxygen as a result of the action of flavoprotein oxidases or nicotinamide adenine hydroxy dinucleotide (NADH) peroxidase. The antimicrobial effect of H_2O_2 may result from the oxidation of sulfhydryl groups causing denaturing of a number of enzymes, and from the peroxidation of membrane lipids thus the increased membrane permeability (Kong and Davison 1980). H_2O_2 may also be as a precursor for the production of bactericidal free radicals such as superoxide (O_2^-) and hydroxyl (OH^-) radicals which can damage DNA (Byczkowski and Gessner 1988).

It has been reported that the production of H_2O_2 by *Lactobacillus* and *Lactococcus* strains inhibited *Staphylococcus aureus*, *Pseudomonas* sp. and various psychotrophic microorganisms in foods (Davidson *et al*. 1983, Cords and Dychdala 1993). In raw milk, H_2O_2 activates the lactoperoxidase system, producing hypothiocyanate ($OSCN^-$), higher oxyacids (O_2SCN^- and O_3SCN^-) and intermediate oxidation products that are inhibitory to a wide spectrum of Gram-positive and Gram-negative bacteria (Reiter and Härnulv 1984, Conner 1993).

Carbon dioxide is mainly produced by heterofermentative LAB. The precise mechanism of its antimicrobial action is still unknown. However, CO_2 may play a role in creating an anaerobic environment which inhibits enzymatic decarboxylations, and the accumulation of CO_2 in the membrane lipid bilayer may cause a dysfunction in permeability (Eklund 1984).

CO_2 can effectively inhibit the growth of many food spoilage microorganisms, especially Gram-negative psychrotrophic bacteria (Farber 1991, Hotchkiss 1999). The degree of inhibition by CO_2 varies considerably between the organisms. CO_2 at 10% could lower the total bacterial counts by 50% (Wagner and Moberg 1989), and at 20-50% it had a strong antifungal activity (Lindgren and Dobrogosz 1990).

2.1.3. Aroma components

Diacetyl is produced by strains within all genera of LAB by citrate fermentation. The antimicrobial effect of diacetyl has been known since the 1930s (Jay 1982). It inhibits the growth of Gram-negative bacteria by reacting with the arginine-binding protein, thus affecting the arginine utilization (Jay 1986).

Jay (1982) showed that Gram-negative bacteria were more sensitive to diacetyl than Gram-positive bacteria; the former was inhibited by diacetyl at 200 µg/mL and the latter at 300 µg/mL. Diacetyl at 344 µg/mL inhibited strains of *Listeria*, *Salmonella*, *Yersinia*, *Escherichia coli*, and *Aeromonas*. Since the production of diacetyl during lactic fermentation is low, e.g. 4 µg/mL produced by *Lc. lactis* ssp. *diacetylactis* (Cogan 1980), and the acceptable sensory levels of diacetyl are at 2-7 µg/mL (Earnshaw 1992), its practical use as a food preservative is limited. However, diacetyl may act synergistically with other antimicrobial factors (Jay 1992) and contribute to combined preservation systems in fermented foods.

Acetaldehyde is produced by *Lb. delbrueckii* ssp. *bulgaricus* by the action of a threonine aldolase, which cleaves threonine into acetaldehyde and glycine. Since *Lb. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* in yoghurt cannot metabolize acetaldehyde, it accumulates in the product at a

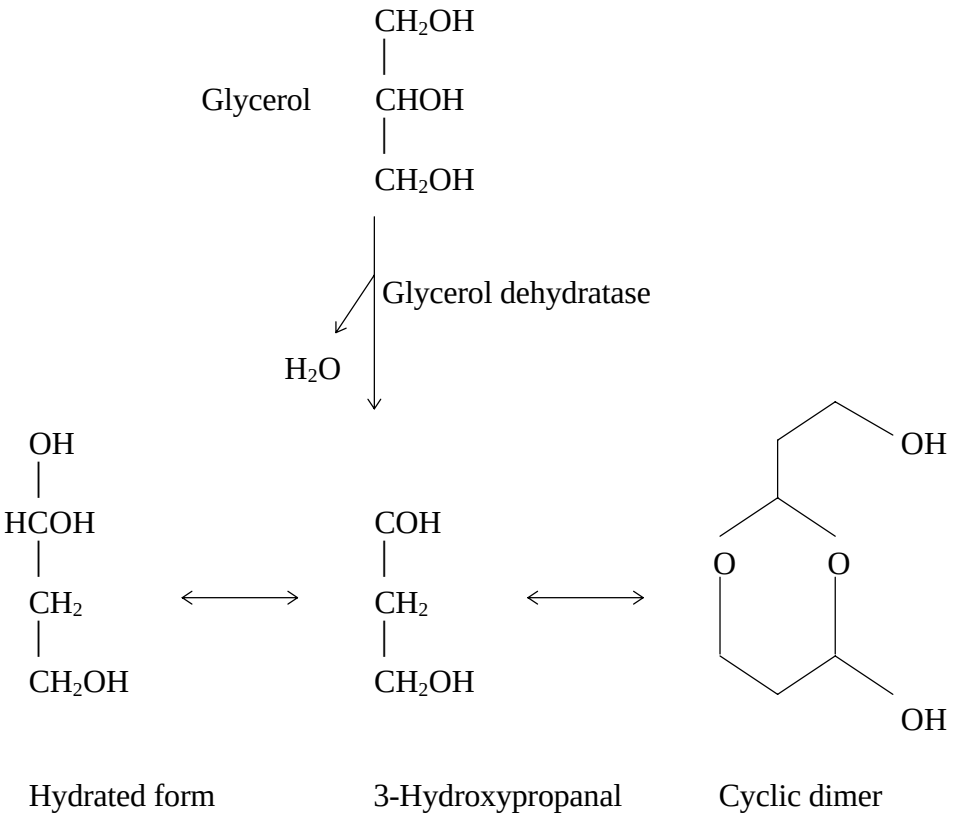
concentration of about 25 ppm. Acetaldehyde at 10-100 ppm inhibits the growth of *Staphylococcus aureus, Salmonella typhimurium* and *E. coli* in dairy products (Piard and Desmazeaud 1991).

2.1.4. Fatty acids

Under certain conditions, some lactobacilli and lactococci possessing lipolytic activities may produce significant amounts of fatty acids, e.g. in dry fermented sausage (Sanz *et al*. 1988) and fermented milk (Rao and Reddy 1984). The antimicrobial activity of fatty acids has been recognized for many years. The unsaturated fatty acids are active against Gram-positive bacteria, and the antifungal activity of fatty acids is dependent on chain length, concentration, and pH of the medium (Gould 1991). The antimicrobial action of fatty acids has been thought to be due to the undissociated molecule, not the anion, since pH had profound effects on their activity, with a more rapid killing effect at lower pH (Kabara 1993).

2.1.5. Reuterin and other low-molecular-mass compounds

Reuterin is produced by *Lb. reuteri*, a heterofermentative species inhabiting the gastrointestinal tract of humans and animals (Axelsson *et al*. 1987). It is formed during the anaerobic growth of *Lb. reuteri* by the action of glycerol dehydratase which catalyzes the conversion of glycerol into reuterin (Talarico *et al*. 1988). Reuterin has been chemically identified to be 3-hydroxypropanal (β-hydroxypropionaldehyde), a highly soluble pH-neutral compound which is in equilibrium with its hydrated monomeric and cyclic dimeric forms (Axelsson *et al*. 1989, Talarico and Dobrogosz 1989). The biosynthesis pathway from glycerol to the three forms of reuterin is shown below.



Reuterin exhibits a broad spectrum of antimicrobial activity against certain Gram-positive and Gram-negative bacteria, yeast, fungi and protozoa. Spoilage organisms sensitive to reuterin include species of *Salmonella, Shigella, Clostridium, Staphylococcus, Listeria, Candida*, and *Trypanosoma* (Axelsson *et al*. 1989).

Besides reuterin which has been well studied, there are several reports on the production of LMM antimicrobial compounds by different species of LAB (Table 1). Niku-Paavola *et al*. (1999) showed that *Lb. plantarum* VTT E-78076 produced LMM compounds active against *Pantoea agglomerans* VTT E-90396 and the fungus *Fusarium avenaceum* VTT D-80147. Other LMM compounds that have been reported are often active at low pH and are heat stable with a broad spectrum of activity (Ouweland 1998). However, it is not clear whether the antimicrobial effects are caused by these compounds or due to primary metabolites such as lactic and acetic acids, hydrogen peroxide, etc. Lortie *et al*. (1993) reported the production of LMM inhibitory substances by *Lb. casei* strains, the activity being possibly due to the effects of small antibiotics, peptides, short-chain fatty acids and lactic acid. The final proof of the production of LMM antimicrobial compounds must be the isolation and identification of these compounds.

2.2. Separation, purification and identification of antimicrobial compounds

2.2.1. Chromatographic separation and purification

For structural studies of antimicrobial compounds produced by LAB, the purity of the material is essential. Since samples of biological origin contain various unknown compounds, the use of chromatographic techniques or a combination of several techniques based on different separation principles is usually needed for purification of such samples.

Talarico *et al*. (1988) separated and purified reuterin from other components in a reaction mixture by reversed-phase high-performance liquid chromatography (RP-HPLC) using two C18 columns in series with water as the mobile phase. The LMM antimicrobial compound, acidolin, produced by *Lb. acidophilus* 2181, was separated by methanol and acetone precipitation, and further purified by gel filtration on Sephadex G-25, and by thin-layer chromatography on silica gel to remove lactic acid (Hamdan and Mikolajcik 1974). The crude extract of a LMM antimicrobial compound from *Lb. delbrueckii* ssp. *bulgaricus* 7994 was separated by ethanol precipitation, and purified by RP-HPLC using an ODS-5 column to yield a single strong peak. However, rechromatography of the active fraction under the same conditions revealed two peaks, one of them being active (Abdel-Bar 1987). Pulusani *et al*. (1979) reported the partial purification of LMM antimicrobial compounds produced by a *S. thermophilus* strain by methanol-acetone extraction and gel filtration.

Although there are some reports of the production of LMM antimicrobial compounds by *Lb. delbrueckii* ssp. *bulgaricus* DDS14 (Reddy *et al*. 1984), *Lb. rhamnosus* GG (Silva *et al.* 1987) and *Lb. casei* strains (Lortie *et al*. 1993), these compounds have not been separated and further purified.

2.2.2. Identification of antimicrobial compounds

Antimicrobial compounds can be identified by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), the latter being often used for the determination of molecular mass. The identification of reuterin was performed mainly by proton (^{1}H) and carbon (^{13}C) NMR studies in deuterium oxide (D_2O) and deuterated methanol, and the molecular mass of

Table 1. Low-molecular-mass (LMM) antimicrobial compounds produced by lactic acid bacteria

Producing strain	Growth medium	Antimicrobial compound	Molecular mass (daltons)	Antimicrobial Activity	Reference
Lactobacillus sp.					
casei ssp. *casei* strains	MRS* broth	Mixture of small antibiotics, peptides and organic acids	< 1 000	*E. coli* and *Streptococcus mutans*	Lortie *et al*. 1993
acidophilus 2181	Skim milk	Acidolin	~ 200	Enteropathogenic organisms and sporeformers	Hamdan and Mikolajcik 1974
delbrueckii ssp. *bugaricus* DD14	Skim milk	Bulgarican		Wide spectrum	Reddy *et al*. 1984
delbrueckii ssp. *bulgaricus* 7994	Skim milk	Nonlactic compound possibly with an aromatic group	< 700	*Pseudomonas fragi* and *Staphylococcus aureus*	Abdel-Bar *et al*. 1987
plantarum VTT E-78076	MRS broth	Containing several LMM compounds	< 700	*Pantoea agglomerans* *Fusarium avenaceum*	Niku-Paavola *et al*. 1999
rhamnosus GG	MRS broth	Microcin-like compound	< 1 000	Wide spectrum	Silva *et al*. 1987
reuteri 1063	Glycerol	3-Hydroxypropanal, and its hydrated and dimer forms (reuterin)	74 92 (hydrated form) 148 (dimer)	Wide spectrum	Talarico and Dobrogosz 1989
Streptococcus sp.					
diacetylactis DRC-1	Whey medium	Cationic compounds	100 - 300	*Pseudomonas* strains and *E. coli*	Branen *et al*. 1975
diacetylactis S_1-67/C	Yeast extract dextrose broth	Ninhydrin positive compounds	385	Wide spectrum	Reddy and Ranganathan 1983
thermophilus strain	Skim milk	Containing carbohydrates and amines	< 700	Wide spectrum	Pulusani *et al*. 1979

* MRS: Man-Rogosa-Sharpe.

the cyclic dimer form of reuterin was shown to be 148 by HPLC-MS and GC-MS (Talarico and Dobrogosz 1989).

The active fraction obtained from gel filtration of the culture filtrate of *Lb. plantarum* VTT E-78076 was shown by GC-MS to contain several different LMM compounds (Niku-Paavola *et al.* 1999). Hamdan and Mikolajcik (1974) reported the identification of acidolin produced by *Lb. acidophilus* 2181 by ultraviolet, infrared, ^{1}H NMR spectroscopy and mass spectrometry. Although the molecular mass of acidolin was suggested to be 198 as indicated by the mass spectra, the chemical nature of acidolin is unknown. The molecular masses of two peptides of acidocin J1132 produced by *Lb. acidophilus* JCM 1132 were determined by MS to be 6220 and 6228 (Tahara *et al*. 1996). For other LMM antimicrobial compounds described above (Table 1), there were no reports on the identification of these compounds, though they were characterized to be nonlactic, microcin-like or cationic compound, etc.

2.3. Methods for evaluation of antimicrobial activity

Among many methods available for evaluation of antimicrobial activity (Parish and Davidson 1993), the methods described below have been used for determining the antimicrobial activity of compounds produced by LAB.

2.3.1. The agar diffusion method

The agar diffusion method has long been used for testing antimicrobial activity, and it was first used by Fleming in 1924 (Piddock 1990). The method has been widely used for evaluation of antimicrobial activity, specially for biologically derived compounds. It includes agar well diffusion assay and disc assay. In this test, an antimicrobial compound is applied to an agar plate on a paper disc or in a well. The compound diffuses into agar resulting in a concentration gradient that is inversely proportional to the distance from the disc or well. The size of the inhibition zone around the disc or well is a measure of the degree of inhibition. The results of the test are generally qualitative (Parish and Davidson 1993). The method requires that the indicator organisms must grow rapidly, uniformly, and aerobically. Since highly hydrophobic antimicrobial compounds cannot diffuse in agar, they are not suitable for tests by this method (Piddock 1990).

Silva *et al*. (1987) used an agar well diffusion assay for testing the antimicrobial activity of *Lb. rhamnosus* GG by addition of a 10-fold concentrate of the GG strain or MRS broth to wells (diameter 4 mm) in agar against various anaerobic and facultative bacteria. The activity of an antimicrobial substance produced by *Lb. delbrueckii* ssp. *bugaricus* 7994 was tested quantitatively with a disc assay procedure, using paper assay discs 12.7 or 6.35 mm in diameter wetted with 30 or 10 µl of sample against *Pseudomonas fragi* and *Staphylococcus aureus* (Abdel-Bar *et al.* 1987). The assay methods used for determination of the antimicrobial activity of different species of LAB were slightly different with respect to the sizes of the wells, discs and samples, and the incubation conditions were dependent on the indicator organisms used (Vignolo *et al*. 1995, Ryan *et al*. 1996, Choi and Beuchat 1994, Aktypis *et al.* 1998).

Several modified procedures based on the agar diffusion method have also been used for testing antimicrobial activity of LAB. These procedures include the agar spot test (Daeschel and Klaenhammer 1985), deferred antagonism assay (Barefoot and Klaenhammer 1983), and spot-on-lawn assay (Hastings and Stiles 1991).

2.3.2. The agar and broth dilution methods

Agar and broth dilution methods are used as quantitative methods, suitable for microorganisms with variable growth rate and for anaerobic, microaerophilic microorganisms (Cintas *et al*. 1995). The results are expressed as MIC, which is the lowest concentration of an antimicrobial that prevents growth of a microorganism after a specific incubation period. In this test, an antimicrobial is serially diluted and a single concentration added to a culture tube or plate added with nonselective broth or melted agar medium, which is then inoculated with test organisms and incubated. The MIC is defined as the lowest concentration at which no growth occurs (absence of turbidity) in a medium following incubation (Parish and Davidson 1993). The broth dilution assay has been used for the determination of the antimicrobial activity of reuterin produced by *Lb. reuteri*, and the activity of reuterin was expressed as MIC values or as the maximum dilutions of the reuterin fraction (Talarico *et al*. 1988, Axelsson *et al*. 1989).

2.3.3. The automated turbidometric assay

A turbidometric assay based on automated systems determines the effect of a compound on the growth or death kinetics of a microorganism. It provides information concerning the effect of an antimicrobial that may cause a delayed lag phase or reduced growth rate at concentrations below the MIC. Since the bacterial growth is monitored by measuring the turbidity of the broth medium, the method demands that the instrument be highly sensitive. Growth at levels below log 5.0 CFU/ml may not be detectable (Davidson and Parish 1989).

Skyttä and Mattila-Sandholm (1991) described a quantitative method based on automated turbidometry for assaying antimicrobial activity, which was expressed as area reduction percentage values measured under the growth curve. The method has been used to test the antimicrobial activity of antimicrobial compounds produced by *P. damnosus* and *P. pentosaceus* (Skyttä *et al*. 1993) and *Lb. plantarum* (Niku-Paavola *et al*. 1999).

2.4. Exopolysaccharides produced by LAB

Lactic acid bacteria produce polysaccharides as cell wall components and storage polymers, and also in many species, as a capsule or slime. In the dairy industry, the slime-forming LAB strains have traditionally been used in the production of fermented milk products, e.g. yogurts, Finnish ‘viili’ and Scandinavian ‘långfil’. It has been generally acknowledged that the secreted EPSs by LAB play an important role in the rheological behavior and texture of the products (Sikkema and Oba 1998, De Vuyst and Degeest 1999).

2.4.1. Homopolysaccharides

Homopolysaccharides are a group of polysaccharides composed of one monosaccharide type. Several species of LAB are able to utilize sucrose as a specific substrate to produce dextrans, mutans, and levans (Sutherland 1972).

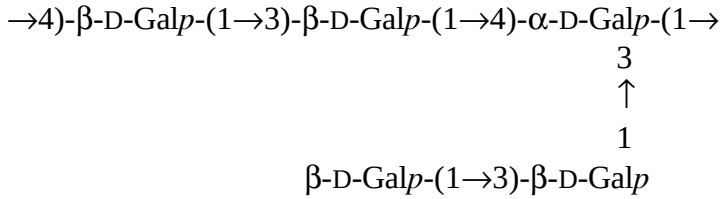
Dextrans are a large class of extracellularly formed glucans produced by the genus *Lactobacillus*, *Leuconostoc*, and *Streptococcus*, of which *Leuc. mesenteroides* and *Leuc. dextranicum* are the well-known dextran producers. Although each bacterial strain produces a unique glucan, a common structural feature of all dextrans is a high percentage (up to 95%) of α-1,6 linkages with a smaller proportion of α-1,2, α-1,3, or α-1,4 linkages resulting in a highly branched molecule (Franz 1986). Dextrans are synthesized outside the cell by dextransucrase, which catalyzes sucrose to produce D-fructose and D-glucose, and transfers the latter to an acceptor to form dextran. The reaction is as follows:

sucrose + glucan acceptor ⟶ dextran or mutans + D-fructose

Mutans are synthesized in a similar way by *S. mutans* and *S. sobrinus* (Montville *et al*. 1978). However, mutans differ from dextrans in containing a high percentage of α-1,3 linkages, which are attributed to the insoluble nature of this type of polymers (Hamada and Slade 1980). Some *S. salivarius* strains are able to produce fructans of the levan type with 2,6-linked β-fructofuranoside residues (Cerning 1990). An extracellular enzyme levansucrase is involved in hydrolyzing sucrose and transferring D-fructose to growing fructan chains to form levans:

sucrose + fructan acceptor ⟶ levan + D-glucose

Another type of homopolysaccharide is the galactan produced by *Lc. lactis* ssp. *cremoris* H414, which is composed of a branched pentasaccharide repeating unit as shown below (Gruter *et al.* 1992).



A *Pediococcus* strain produced a β-D-glucan with a trisaccharide repeating unit (Llaubères *et al*. 1990). *Lactobacillus* spp. G-77 has been shown to produce a 2-substituted-(1→3)-β-D-glucan, identical to the EPS produced by *P. damnosus* 2.6 (Dueñas-Chasco *et al*. 1997). *Lactobacillus* spp. G-77 also produced a α-D-glucan composed of a trisaccharide repeating unit (Dueñas-Chasco *et al*. 1998). Recently, van Geel-Schutten *et al*. (1998) reported for the first time the production of a fructan by *Lb. reuteri* strain LB121 with raffinose as a sugar substrate; this strain also produced both a glucan and a fructan on sucrose.

2.4.2. Heteropolysaccharides

A wide range of LAB strains can produce heteropolysaccharides, which are composed of repeating units. The monosaccharide compositions of these EPSs are mostly galactose and glucose, and also small amounts of rhamnose, fructose, mannose, and galactosamine (van den Berg *et al*. 1995). In comparison with the homopolysaccharides, the production of heteropolysaccharides by LAB is much lower (60 to 400 mg L^{-1}) (Stingele *et al*. 1996). Generally, the heteropolysaccharides are synthesized intracellularly at the cytoplasmic membrane utilizing sugar nucleotides as precursors for the assembly of polysaccharide chains (Cerning 1995). Table 2 lists the heteropolysaccharides produced by the genera *Lactobacillus*, *Lactococcus* and *Streptococcus*.

2.4.2.1. *Lactobacillus*

The ability of lactobacilli to produce EPSs has been recognized for many years. In 1968, Kooiman (from Sikkema and Oba 1998) first reported the structure of a heteropolysaccharide

Table 2. Heteropolysaccharides produced by lactic acid bacteria

Strain	Growth medium	EPS production (mg L^{-1} culture)	Molecular mass	EPS composition *				Repeating unit	Reference
				Gal	Glc	Rha	Others		
Lactobacillus									
acidophilus LMG9433	SDM			1	2		1 GlcA 2 GlcNAc	pentamer	Robijn *et al*. 1996c
casei CG11	BMM	160		1	17	3			Cerning *et al*. 1994
casei NCIB 4114	skim milk			2.5	5	1			Cerning *et al*. 1992
casei CRL 87	skim milk	121	7.9×10^5	1		1.1			Mozzi *et al*. 1995, 1996
delbrueckii ssp.									
bulgaricus CNRZ 416	skim milk	285	4.9×10^5	4	1	1			Cerning *et al*. 1986
bulgaricus CNRZ 737	skim milk	424		4	1	1			Cerning *et al*. 1986
bulgaricus CNRZ 1187	skim milk	110		14	9		1 Man		Bouzar *et al*. 1996
bulgaricus CRL 420	skim milk		2.0×10^5		1		2 Fru		Manca de Nadra *et al*. 1985
bulgaricus NCFB 2772	CDM	37	1.5×10^6	6.8	1	0.7			Grobben *et al*. 1995
bulgaricus OLL1037R-1	skim milk	58	1.2×10^6	3	2				Uemura *et al*. 1998
			1.1×10^6	3	2				
bulgaricus rr	SDM	354		5	1	1		heptamer	Gruter *et al*. 1993; Kimmel *et al*. 1998
helveticus	whey medium			1	2				Oda *et al*. 1983
helveticus 766	skim milk			1	2			hexamer	Robijn *et al*. 1995a
helveticus TY 1-2	skim milk	200	1.6×10^6	2.8	3		1 GlcNAC	heptamer	Yamamoto *et al*. 1994
helveticus TN-4	skim milk	180	$1..8 \times 10^6$	1	1			hexamer	Yamamoto *et al*. 1995
helveticus Lh59	skim milk	272	2.0×10^6	1	1			hexamer	Stingele *et al*. 1997
kefiranofaciens K_1	IKPL	63	1.0×10^6	1.1	0.9			hexamer	Mukai *et al*. 1990
paracasei 34-1	SDM			3			1 GalNAc 1 G3P	tetramer	Robijn *et al*. 1996a
rhamnosus strain C83	BMM	132		3	2			pentamer	Gamar *et al*. 1997, Vanhaverbeke *et al*. 1998
sake 0-1	SDM	1400	6.0×10^6		3	2	1 G3P 0.85 Ac	pentamer	van den Berg *et al*. 1995, Robijn *et al*. 1995b
Lactococcus lactis ssp.									
cremoris B 40	whey medium			2	2	1	1 P	pentamer	van Casteren *et al*. 1998
cremoris SBT 0495	whey medium	150	1.7×10^6	2	2	1	1 P	pentamer	Nakajima *et al*. 1990, 1992a

cremoris LC330	cremoris defined medium	25	2.0×10^6	3	6		2 GlcNAc			Marshall *et al*. 1995
			1.0×10^4	4	6	5	1 GlcNAC	1 P		
cremoris T_5	skim milk	600		1.2	1					Cerning *et al*. 1992
cremoris MLS96	skim milk	220		1	1					Cerning *et al*. 1992
Streptococcus salivarius ssp.										
thermophilus CNRZ 389	skim milk	57		11	4.8	1	3 Man			Cerning *et al*. 1988
thermophilus CNRZ 1068	skim milk	166		1.2	1					Cerning *et al*. 1988
thermophilus CNCMI 733, 734, 735	skim milk	42	1.0×10^6	2	1		1 GalNAC		tetramer	Doco *et al*. 1990
thermophilus LY03	skim milk	546		4	1					De Vuyst *et al*. 1998
thermophilus MR-1C	M17 broth			5		2	1 Fuc			Low *et al*. 1998
thermophilus OR 901	whey medium			5		2			heptamer	Bubb *et al*. 1997
thermophilus Rs	skim milk	135	2.6×10^6	5		2			heptamer	Faber *et al*. 1998
thermophilus Sts	skim milk	127	3.7×10^6	5		2			heptamer	Faber *et al*. 1998
thermophilus Sfi 6	skim milk + amino acids	175	2.0×10^6	2	1		1 GalNAc		tetramer	Stingele *et al*. 1996
thermophilus Sfi12	skim milk + amino acids	105	$> 2 \times 10^6$	3	1	2			hexamer	Lemoine *et al*. 1997
thermophilus Sfi39	skim milk + amino acids	350	$> 2 \times 10^6$	1	1				tetramer	Lemoine *et al*. 1997

Ac: acetyl; BMM: basal minimum medium; CDM: chemically defined medium; Fru: fructose; Fuc: fucose; Gal: galactose; GalNAc: *N*-acetylgalactosamine. Glc: glucose; GlcNAc: *N*-acetylglucosamine; G3P: sn-glycerol-3-phosphate; Man: mannose; P: phosphate; Rha: rhamnose; SDM: semi-defined medium.
* Part of the data is compiled from Sikkema and Oba (1998).

produced by a *Lb. brevis* strain isolated from kefir grains. This polysaccharide consists of a hexasaccharide repeating unit with D-galactose and D-glucose in the molar ratio 1:1. In the last decade, a number of heteropolysaccharides produced by the *Lactobacillus* species have been investigated.

Lb. helveticus strains produce several EPSs with varying repeating units, though all containing galactose and glucose (Fig. 1). The EPS produced by *Lb. helveticus* 776 has hexasaccharide repeating units containing D-galactose and D-glucose (Robijn *et al*. 1995a). The EPS produced by *Lb. helveticus* TY1-2 consists of heptasaccharide repeating units with D-galactopyranosyl and D-glucopyranosyl, and 2-acetamido-2-deoxy-D-glucopyranosyl residues (Yamamoto *et al*. 1994). Recently, Stingele *et al*. (1997) showed that the EPS produced by *Lb. helveticus* Lh59 had an identical primary molecular structure as the one produced by *Lb. helveticus* TN-4 (Yamamoto *et al*. 1995), a presumed spontaneous mutant of the strain TY1-2. This polymer is composed of a tetrasaccharide backbone with a lactosyl side-chain, and the molar ratio of D-galactose and D-glucose is 1:1.

A

β-D-Gal*f*
1
↓
3
→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→

B

(α-D-Gal*p*)$_{0.8}$
1
↓
4
→6)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→6)-α-D-Glc*p*NAc
1
↓
3
β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-β-D-Gal*p*-(1→

C

β-D-Gal*p*-(1→4)-β-D-Glc*p*
1
↓
3
→3-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→5)-β-D-Gal*f*-(1→

Fig. 1. Structures of the repeating units of exopolysaccharides (EPSs) produced by four *Lb. helveticus* strains: A. strain 766 (Robijn *et al.* 1995b); B. strain TY1-2 (Yamamoto *et al.* 1994); C. strains TN-4 (Yamamoto *et al*. 1995) and Lh59 (Stingele *et al*. 1997).

Robijn *et al*. (1995b) reported the primary molecular structure of a viscous EPS produced by *Lb. sake* 0-1 which was isolated from fermented meat products. The EPS consists of a pentasaccharide repeating unit of glucose, rhamnose, and glycerol phosphate. The three-dimensional structure of this polymer has been further studied by molecular mechanics calculations (Robijn *et al.* 1996b). The helics generated by a polysaccharide builder program are highly extended, with either 2-fold or 3- or 4-fold right-handed chiralities.

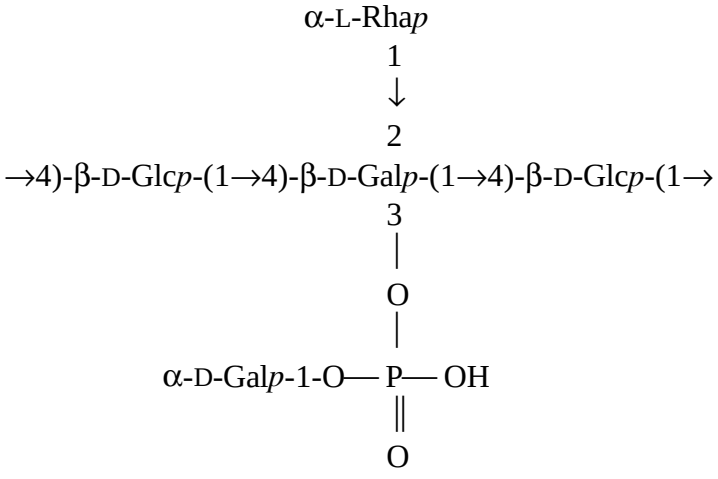
Grobben *et al*. (1997) showed that *Lb. delbrueckii* ssp. *bulgaricus* NCFB 2772 produced an EPS made up of galactose, and small quantities of glucose and rhamnose, and another EPS that, according to Sikkema and Oba (1998), was similar to the structure of the EPS produced by *Lb. delbrueckii* ssp. *bulgaricus* rr (Gruter *et al*. 1993). The enzymes involved in the production of the sugar nucleotides of strain NCFB 2772 have been analyzed, and based on this analysis a biosynthetic pathway for the EPS has been proposed (Grobben *et al*. 1996). Growth of the strain in a fructose-based medium led to the absence of the enzyme activities for the synthesis of the rhamnose nucleotide, and accordingly no rhamnose was present in the polysaccharide produced.

The EPSs produced by *Lb. acidophilus* LMG9433 (Robijn *et al*. 1996c), *Lb. kefiranofaciens* K_1 (Mukai *et al*. 1990), and *Lb. paracasei* 34-1 (Robijn *et al*. 1996a) have also been structurally evaluated, the repeating units being pentamers, hexamers and tetramers, respectively. Recently, *Lb*. *rhamnosus* strain C83 has been shown to produce an EPS composed of a pentasaccharide repeating unit with a linear structure (Vanhaverbeke *et al*. 1998). This strain, as well as *Lb. casei* CG11 (Cerning *et al.* 1994) and *Lb. sake* 0-1 (van den Berg *et al*. 1995), produced more EPSs at lower temperatures, whereas several other *Lactobacillus* strains produced more EPSs at higher temperatures (compared with the optimum temperatures of growth) (Grobben *et al*. 1995, Garcia-Garibay and Marshall 1991).

2.4.2.2. *Lactococcus*

Among lactococci, only the slime-forming *Lc. lactis* ssp. *cremoris* strains have been investigated. These strains producing EPSs play a role in the proper consistency of the fermented milk (Cerning 1995). The sugar components of the EPSs are most frequently galactose, glucose, and very often rhamnose (Cerning 1990).

Nakajima *et al.* (1992a) reported a phosphate-containing heteropolysaccharide, named ‘viilian’, produced by *Lc. lactis* ssp. *cremoris* SBT 0495 which was isolated from a Finnish ‘viili’ starter culture. The EPS consists of the following repeating unit:



Lc. lactis ssp. *cremoris* B40 has also been found to produce a phosphopolysaccharide with an identical repeating unit as shown above (van Casteren *et al*. 1998). *Lc. lactis* ssp. *cremoris* strain LC330 appeared to produce concurrently two EPSs; an anionic EPS composed of galactose, glucose, rhamnose, glucosamine and phosphate, and a neutral EPS containing galactose, glucose and glucosamine with branched terminal galactose moieties (Marshall *et al.* 1995). The mechanism for the biosynthesis of the EPS by *Lc. lactis* ssp. *cremoris* has not been investigated in detail. Recently, Oba *et al.* (1996) proposed a biosynthetic pathway for the production of 'viilian' by strain SBT 0495 with the following steps: preparation of the membrane-embedded lipid carrier; incorporation of the first monosaccharide with the phosphate on C-1; assembly of the intact repeating unit.

The biosynthesis of the EPSs produced by *Lc. lactis* strains is generally associated with a plasmid (Kleerebezem 1999). Transferring mucoidity plasmids from *Lc. lactis* ssp. *cremoris* ARH87 and MS to nonmucoid *Lc. lactis* strains proved the latter to be mucoid (Vedamuthu and Neville 1986, von Wright and Tynkkynen 1987). van Kranenburg *et al*. (1997) described a novel 12 kb EPS gene cluster located on a 40 kb plasmid, which was essential for the EPS synthesis of *Lc. lactis* ssp. *cremoris* NIZO B40. Introduction of the EPS gene cluster from *S. thermophilus* Sfi6 to a non-EPS-producing *Lc. lactis* MG1363 produced an EPS with a different structure from the EPS of the native host (Stingele *et al*. 1999). The absence of the GalNAc residue in the EPS of *Lc. lactis* MG1363 was probably caused by the lack of a UDP-*N*-acetylglucosamine C4-epimerase activity (Fig. 2).

A. →3)-β-D-Glc*p*-(1→3)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*-(1→
6
↑
1
α-D-Gal*p*

B. →3)-β-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→

Fig. 2. Structures of the EPSs produced by: A. *S. thermophilus* Sfi6; B. *Lc. lactis* MG1363 (Stingele *et al*. 1999).

2.4.2.3. *Streptococcus*

S. thermophilus strains are used in combination with *Lb. delbrueckii* ssp. *bulgaricus* strains in yoghurt starters. The EPSs produced by several *S. thermophilus* strains have been found to have similar or identical primary molecular structures.

Doco *et al*. (1990) first reported the structure of an EPS produced by ropy *S. thermophilus* strains CNCMI 733, CNCMI 734 and CNCMI 735, which consisted of a tetrasaccharide repeating unit of D-galactose, D-glucose, and *N*-acetyl-D-galactosamine in a molar ratio 2:1:1. An EPS with an identical repeating unit structure has been reported to be produced by *S. thermophilus* Sfi6 (Stingele *et al*. 1996). Lemoine *et al*. (1997) showed that the EPSs produced by *S. thermophilus* Sfi12 and Sfi39 had molecular masses greater than 2 x 10^6, and both yielded a slimy texture rather than a thickened one in yoghurt. However, they had different sugar compositions and structures; the former consisting of a hexasaccharide repeating unit of

galactose, glucose and rhamnose, and the latter a tetrasaccharide repeating unit of galactose and glucose.

Recently, Faber *et al*. (1998) showed that *S. thermophilus* Rs and Sts produced EPSs of identical repeating units, but they had different molecular masses, resulting in a difference in viscosity in their milk cultures. Bubb *et al*. (1997) also showed that the EPS produced by *S. thermophilus* OR 901 had a similar repeating unit to the one of the strains Rs and Sts; all being branched heptasaccharide repeating units of D-galactose and L-rhamnose in the same molar ratio 5:2.

2.5. Isolation and structural elucidation of EPSs

2.5.1. Isolation of EPSs

EPSs produced by LAB can be isolated by precipitation with trichloroacetic acid (TCA) (Gruter *et al.* 1992) to remove proteins from the culture media, and subsequently by ethanol (Faber *et al*. 1998) and/or acetone (Lemoine *et al*. 1997) to precipitate polysaccharides. Gel filtration or ion exchange chromatogrphy is often used for further purification of EPSs. Robijn *et al*. (1995a, 1995b, 1996a, 1996b) purified EPSs produced by several *Lactobacillus* strains by gel filtration with different columns (Sephacel 500, Sephacryl S-500, and Superrose-6). Gel filtration techniques were also used for purification of the EPSs produced by *Lc. lactis* ssp. *cremoris* H414 (Gruter *et al.* 1992), *S. thermophilus* Sfi16 (Stingele *et al.* 1996), and *S. thermophilus* CNCMI 733 (Doco *et al*. 1990). Since many EPSs are negatively charged, they can be bound to an anion exchanger. This technique has been used for purification of the anionic EPSs produced by *Lc. lactis* ssp. *cremoris* B40 (van Casteren *et al*. 1998), and *Lb. helveticus* TY1-2 (Yamamoto *et al*. 1994) and TN-4 (Yamamoto *et al*. 1995). Marshall *et al*. (1995) showed that *Lc. lactis* ssp. *cremoris* strain LC330 produced at the same time both neutral and anionic EPSs in the medium; these two EPSs were effectively separated and purified by anion exchange chromatography.

2.5.2. Sugar and methylation analyses

In the analysis of monosaccharide compositions of EPSs produced by LAB, the EPSs are hydrolyzed with trifluoroacetic acid (TFA), reduced and acetylated, and the acetate derivatives are analyzed with GC. For the methylation analysis, monosaccharides are usually derivatized into partially methylated alditol acetates, which are introduced into the EI source of MS from a GC, or GLC interface. The substitution pattern of the monosaccharides can be determined by comparing their fragmentation pattern with reference EI-MS spectra. Yamamoto *et al.* (1994, 1995) studied the substitution pattern of the monosaccharides in the EPSs produced *Lb. helveticus* TY1-2 and TN-4 by GLC-MS of the methylated and acetylated sugar residues. The EPS produced by *Lb. helveticus* 766 was analyzed by GLC-MS on DB-1 of the partially methylated alditol acetates (Robijn *et al*. 1995a).

2.5.3. Nuclear magnetic resonance spectroscopy

NMR spectroscopy relies on the interaction of radio-frequency electromagnetic radiation with magnetically active nuclei in a strong magnetic field. The radio frequencies used range from 200 to 800 MHz, corresponding to magnetic fields from 4.7 to 18.8 Tesla. ^{1}H and ^{13}C are the spin-active nuclei most frequently encountered in carbohydrates. ^{1}H and ^{13}C NMR spectroscopy,

including one- (1D) and two-dimensional (2D), is a powerful tool for structural studies of carbohydrates (Widmalm 1998), which also include polysaccharides produced by LAB.

1D ^{1}H NMR spectroscopy can be used for rapid identification or to check the purity of a polysaccharide sample. Signals in the anomeric region (about 4.3-5.5 ppm) of the spectrum and the coupling of the anomeric protons ($J_{H1,H2}$) may provide useful information about the number of residues in a repeating unit, and the anomeric configuration, respectively. Yamamoto *et al.* (1995) recorded the 500 MHz ^{1}H NMR spectrum of the EPS produced by *Lb. helveticus* TN-4 in D_2O at 70 ^{o}C, and found six signals in the anomeric region with nearly equal integrated intensities, suggesting there was a hexasaccharide repeating unit for this EPS. A study on the EPS produced by *Lb. helveticus* Lh59, by 400 MHz ^{1}H NMR spectroscopy in Me_2SO-d_6 at 80 ^{o}C produced four anomeric protons signals in a molar ratio 1:2:2:1, which also indicated a hexasaccharide repeating unit (Stingele *et al*. 1997). The ^{1}H NMR spectrum of the EPS produced by *Lb. paracasei* had four doublets in the anomeric region, and the coupling constants ($J_{H1,H2}$) of these signals (8.3 Hz, 7.7 Hz, 7.3 Hz, 7.7 Hz) were of the pyranoid ring form with all the residues in the β configuration (Robijn *et al*. 1996a).

Since the natural abundance of ^{13}C is very low (1.1% relative to ^{12}C), the peak intensity of ^{13}C has to be enhanced in 1D ^{13}C NMR spectroscopy by using a large number of pulses, by taking advantage of the nuclear Overhauser effect (NOE), or by using distortionless enhancement by polarization transfer (DEPT) experiments. The values of ^{13}C chemical shift and ^{13}C-^{1}H coupling ($^{1}J_{C,H}$) provide structural information of the polysaccharides. Robijn *et al*. (1995a) found six signals in the anomeric region of the ^{13}C NMR spectrum of the EPS produced by *Lb. helveticus* 766, confirming the suggested hexasaccharide repeating unit by ^{1}H NMR spectroscopy. Based on the C-1 chemical shifts in the ^{13}C NMR spectrum of the EPS produced by *Lb. rhamnosus* strain C83, Vanhaverbeke *et al.* (1998) assigned two downfield signals (δ 110.12, 107.67) to two residues having β configuration, and three signals at δ 99.73, 99.99 and 100.73 to three residues having α configuration.

The 1D NMR techniques are often used for the assignment of signals in the anomeric region. For detailed assignment for the spin system of sugar residues, 2D techniques are needed. These techniques include ^{1}H,^{1}H-correlated spectroscopy (COSY), total correlation spectroscopy (TOCSY), homonuclear Hartmann-Hahn spectroscopy (HOHAHA), gradient selected heteronuclear single quantum coherence (gHSQC), gradient selected heteronuclear multiple-bond correlation (gHMBC) experiment, and nuclear Overhauser effect spectroscopy (NOESY). Bubb *et al*. (1997) used a TOCSY experiment to further assign two signals of anomeric protons in the ^{1}H NMR spectrum of the EPS produced by *S. thermophilus* OR 901. By means of 2D COSY, HOHAHA, NOESY, and ^{13}C-^{1}H HMQC (heteronuclear multiple quantum coherence), Robijn *et al*. (1996a) assigned almost all ^{1}H and ^{13}C resonances in 1D ^{1}H and ^{13}C NMR spectra of the EPS produced by *Lb. paracasei* 34-1.

The sequence of the monosaccharide residues in a repeating unit can be established by 2D NOESY and HMBC experiments. The former experiment gives information about the inter-residue linkage from observation of the NOE between anomeric protons and the protons at the substituted positions of neighbouring sugar residues. The latter experiment gives rise to cross-peak between proton and carbon atoms that are long-range scalar coupled. Faber *et al.* (1998) used 2D NOESY together with HSQC-NOE experiments to determine the sequence of the sugar residues in the EPS produced by *S. thermophilus* Rs and Sts. The monosaccharide sequence in the EPS produced by *Lb. paracasei* 34-1 was established by 2D NOESY experiments (Robijn *et al*. 1996a).

2.6. Rheological characterization of EPSs

2.6.1. Solution viscosity

Viscosity η is defined as the ratio between shear stress and shear rate. The intrinsic viscosity [η], which measures the hydrodynamic volume of a molecule, is obtained by extrapolating the Huggins equation to zero concentration: $\eta_{sp}/c = [\eta] + k'[\eta]^2 c$, where η_{sp} is specific viscosity, c is polymer concentration, and k' is a constant for a series of polymers of different molecular mass in a given solvent. η_{sp}/c is also defined as the reduced viscosity η_{red}.

For ionic polysaccharides in aqueous solutions, the value of η_{red} increases with decreasing concentration, showing a polyelectrolyte effect. The behavior of the polyelectrolytes is influenced by intrachain Coulombic interaction, ionic strength, pH and specific counterions (Paoletti 1998). Oba *et al*. (1999) suggested that in a strain sweep test at very high dilution of the EPS produced by *Lc. lactic* ssp. *cremoris* SBT 0495, the higher cross-over frequency of the EPS in 0.1 M NaCl compared to that in pure water was due to the polyelectrolyte effect of this EPS. van den Berg *et al*. (1995) showed that over a wide range of shear rates, the viscosity of a 1% solution of the EPS produced by *Lb. sake* 0-1 decreased with increasing shear rates, indicating a shear-thinning behavior, and the viscosity was comparable to that of xanthan gum.

2.6.2. Dynamic viscoelasticity

In response to an applied stress, polysacharides may show a viscoelastic behavior, i.e. a combination of truely viscous flow and perfectly elastic response. In a dynamic test, the polysaccharide sample is subject to sinusoidal shear oscillation with a wide range of frequencies (0.01-300 Hz). The relative magnitudes of G' (storage modulus) and G" (loss modulus) vary with the state of the polysaccharide. For entangled solutions, where there is a greater contribution from the viscous element, G' is low. When frequency decreases, there is a crossover in G' and G", and they flow as high viscosity liquids at very low frequencies. For gel systems, G' and G" are parallel, with G' > G" and largely frequency independent (Ross-Murphy 1998).

Oba *et al*. (1999) showed that in a dynamic and steady shear measurement the aqueous solutions of the EPS produced by *Lc. lactis* ssp. *cremoris* SBT 0495 behaved as an entangled solution but not as a weak gel. Nishinari (1997) reported the frequency (0.01 - 10 ω/rad·s^{-1}) dependence of G' and G" for 1-3% solutions of gellan gum, an EPS produced by *Pseudomonas elodea*. The 1% (0-30 °C) and 2% (30 °C) solutions had a typical dilute solution behavior with G" > G'. The 2% (15 °C, 25 °C) and 3% (30 °C) solutions, however, had a concentrated solution behavior with a crossover of G' and G" and G' > G" at higher frequencies. Gelation occurs at 3% at 0-25 °C with G' and G" being slightly frequency dependent.

2.6.3. Gelling properties

Gels are defined as loose three-dimensional networks with structures ranging from homogeneous solutions (enthalphy-driven aggregations) to heterogeneous rigid porous systems (Li *et al.* 1996). Clark and Farrer (1995) described the mechanisms of gel formation in three main classes, firstly by point crosslinking with covalent bonds, secondly by chain association driven by changes in temperature, pH and ionic strength, and the presence of small molecules and specific counterions, and thirdly by particle aggregation. Many polysaccharide gels are formed by thermoreversable physical associations, involving Coulombic, dipole-dipole, van der Waals,

charge transfer, and hydrophobic and hydrogen bonding interactions, as well as double-helix formation and aggregation (Guenet 1992).

Gels can be characterized to be strong or weak based on their response to deformation. At large deformations, strong gels will rupture and fail, while weak gels flow without fracture, and show recovery of solid character. Xanthan gum, the EPS produced *by Xanthomonas campestris*, forms a weak gel, with large deformation fluid properties, but it also forms strong gel under extreme conditions (Ross-Murphy 1995).

2.6.4. Rheological behaviors of EPSs in fermented milk

The use of EPS-producing LAB strains may improve the rheological properties of fermented milk. The gel structure and viscosity of the products are affected by the gel formation conditions, as well as the amount and the type of the EPSs produced.

Hammelehle *et al*. (1998) showed that fast warming rates (20-50 °C) during acidification increased the gel firmness and storage modulus, and decreased the syneresis of a milk gel. Skim milk fermented by ropy EPS-producing strains exhibited similar rheological properties, and had greater viscosity than skim milk fermented by non-ropy strains (Schellhaass and Morris 1985). Ropy EPS-producing strains also increased the viscosity of yoghurt when compared to yoghurt made with non-ropy cultures (Rawson and Marshall 1997, Sebastiani and Zelger 1998), and improved the texture of quarg (Sebastiani *et al*. 1997). As described ealier, *S. thermophilus* Rs and Sts produced EPSs of the same structure, but had different viscosities in the milk cultures due to their different molecular masses (Faber *et al*. 1998). The rheological behavior of the polysaccharides is also related to their three-dimensional structure (Robijn *et al*. 1996b).

In addition to the viscosifying effect of the polysaccharides, the interactions between the EPSs and the milk proteins, e.g. caseins, also play a role. Studies of a yogurt gel with a scanning electron microscopy showed that the cells were attached to the protein coagulates by a network structure consisting of polysaccharide filaments (Schellhaass and Morris 1985, Toba *et al*. 1990). The microorganisms and/or the EPSs that they produce may affect the protein aggregation, thereby affecting the physical properties of the milk gel (van Marle and Zoon 1995). A recent study showed that the rheological properties of stirred yoghurt were affected by the type of EPS-producing strains used, suggesting an effect due to the interaction between the polymer and milk proteins (Marshall and Rawson 1999). Hess *et al.* (1997) proposed a model for shear-induced degradation of the microstructure of EPS-producing yogurt. Since the associations of EPS with bacterial cells or casein micelles are stronger than the associations between the casein micelles, an increase in shear will first disrupt the casein micelle network that is not associated with EPS, subsequentely the associations between the cells and EPS, and then the portion of the casein network that is associated with EPS.

2.7. Applications in foods and health aspects

2.7.1. Antimicrobial compounds as natural food preservatives

The quality of most foods deteriorates during storage. In addition to physical, chemical and enzymatic factors which may alter the sensory characteristics, the microbiological changes in foods may bring about a wide range of spoilage reactions, including food poisoning (Gould 1991). Therefore, it is of significance to inhibit the growth of spoilage microorganisms in foods. Due to a strong demand for natural and minimally processed foods, there has been a growing

interest in the use of antimicrobial compounds produced by LAB as a safe and natural way of food preservation.

In addition to nisin which has been widely used in foods (Qiao 1996), another antimicrobial compound that has been proposed for use in food preservation is reuterin produced by *Lb. reuteri* (Lindgren and Dobrogosz 1990). Addition of reuterin to ground beef was found to inhibit the growth of *E. coli*. (Daeschel 1989). Surface treatment of herring with a mixture of *Lb. reuteri* and glycerol significantly improved the shelf-life of the product (Lindgren and Dobrogosz 1990). *Lb. reuteri* has been commercially used in combination with *Bifidobacterium infantis* and *Lb. acidophilus* in sweet and fermented milk under the trade name BRA-mjölk (Rothschild 1995).

Antimicrobial compounds can be applied to foods either as purified chemical agents, or as viable cultures in the case of fermented products (Barnby-Smith 1992). Novel purified antimicrobial compounds require data to substantiate their lack of toxicity in order to obtain approval for their use in foods. Traditional fermented products that naturally contain antimicrobial compounds have been consumed for centuries, and starter cultures with selected antimicrobial properties may be used to replace those used in traditional fermented foods. However, problems may arise with respect to retaining the flavor and texture of the products (Earnshaw 1992).

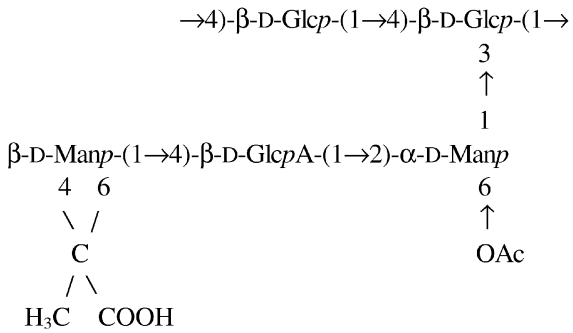
2.7.2. EPSs in food applications

Polysaccharides may function in foods as viscosifying agents, stabilizers, emulsifiers, gelling agents, or water-binding agents (van den Berg *et al.* 1995). The majority of the polysaccharides used in foods are of plant origin. Most of them are chemically or enzymatically modified in order to improve their rheological properties, e.g. cellulose, starch, pectin, alginate and carrageenan. Therefore, their use is strongly restricted. EPSs of microbial origin have unique rheological properties because of their capability of forming very viscous solutions at low concentrations and their pseudoplastic nature (Becker *et al.* 1998). The EPSs produced by food-grade LAB have been considered as a new generation of food thickeners to improve the rheological properties of foods (Robijn 1996).

Dextran is the first industrial polysaccharide produced by LAB. It was discovered in 1880 in sugar cane or beet syrups where dextran was found to be responsible for the thickening and gelation of the syrups (Crescenzi 1995). Due to their structural differences, some dextrans are water soluble and others are insoluble. Dextran can be used in confectionary to improve moisture retention, viscosity and inhibit sugar crystallization. In gum and jelly candies it acts as a gelling agents. In ice cream it acts as a crystallization inhibitor, and in pudding mixes it provides the desirable body and mouth feel (Whistler and Daniel 1990). In addition, dextran has also been used as blood plasma extenders and as the basic component of many chromatographic stationary phases (Franz 1986).

Xanthan gum is the second microbial EPS which was approved for use in foods in 1969. Although it is produced by the plant-pathogen *Xanthomonas campetris*, Sutherland (1998) described xanthan as the "benchmark" product with respect to its importance in both food and nonfood applications, which include dairy products, drinks, confectionary, dressing, bakery products, syrups and pet foods, as well as the oil, pharmaceutical, cosmetic, paper, paint and textile industries. The production of xanthan is relatively inexpensive because of the high conversion of substrate (glucose) to polymer (60-70%) (Sutherland 1998). According to Becker *et al*. (1998), xanthan in solutions exhibits a high viscosity at low concentrations and strong pseudoplasticity, and it is stable over a wide range of pHs, temperatures and ionic strengths.

Xanthan also forms a weak gel, and synergistic interactions with galactomannans or glucomannans leading to strong gels (Urlacher and Noble 1997). It has been suggested that the unique rheological properties of xanthan result from its rigid, rod-like conformation adopted in solution, in which the trisaccharide side chains align with the cellulose backbone (Sutherland 1998). The primary molecular structure of the repeating unit of xanthan is shown below (Jansson *et al.* 1975, Melton *et al.* 1976).



In order to find novel EPSs for food applications, van den Berg *et al.* (1993) screened 600 LAB strains, and found 30 EPS-producers. One of the producers was *Lb. sake* 0-1, which produced an EPS with high viscosity comparable to that of xanthan, and a high yield (1.4 g L^{-1}) (van den Berg *et al.* 1995). The authors considered this EPS to have potential applications in a variety of food products. Recently, van Geel-Schutten *et al.* (1998) screened a total of 182 *Lactobacillus* strains in order to produce large amounts of EPSs, and suggested that sucrose was an excellent substrate for abundant EPS synthesis. A recent trial by Low *et al.* (1998) showed that the use of an EPS-producing starter pair, *S. thermophilus* MR-1C and *Lb. delbrueckii* ssp. *bulgaricus* MR-1R, significantly increased moisture retention in low-fat Mozzarella (cheese). A capsular EPS produced by *S. thermophilus* MR-1C was found to be responsible for the water-binding properties of this bacterium, and the EPS was shown to consist of D-glucose, L-rhamnose, and L-fucose in a molar ratio of 5:2:1.

Although the production of EPSs by LAB is positively related to the rheological properties of food products, it may also be disadvantageous in food processing (De Vuyst and Degeest 1999). In the sugar industry, dextran and levan may cause sucrose loss and filtration problems. Their biofilm formation in heat-exchangers may contaminate dairy products. The adhesion of thermophilic bacteria to the heat-exchangers may especially contaminate already pasturized milk. In addition, slime-forming *Pediococcus* strains may cause the spoilage of beers, wines, ciders and meat products.

2.7.3. Health aspects

In the last decade there has been an increasing interest in using LAB as probiotics. The important criteria for a LAB strain to be a probiotic are, in addition to acid and bile tolerance, the ability to produce antimicrobial compounds against pathogenic and cariogenic bacteria, and to adhere to and colonize human intestinal mucosa (Saxelin 1995).

The production of antimicrobial compounds may be beneficial to the colonization of probiotics in gut mucosa by increasing their competitive advantage against normal gastrointestinal

microflora (Salminen *et al*. 1998a). The probiotic *Lb. rhamnosus* GG produced a LMM microcin-like compound, inhibitory toward *Bacteroides*, *Bifidobacterium*, *Clostridium*, *E. coli*, *Pseudomonas*, *Salmonella*, and *Streptococcus* (Silva *et al.* 1987). Another probiotic *Lb. reuteri* also produced an antimicrobial compound with a wide spectrum of activities (see 2.1.5.).

Studies on bacterial adhesion showed that capsular polysaccharide might promote the adherence of bacteria to biological surfaces, thereby facilitating the colonization of various ecological niches (Costerton *et al*. 1987). The EPSs were found to be present in adherent biofilms (Whitfield 1988); the EPSs might function as initial adhesion, and permanent adhesion compounds (Allison and Sutherland 1987).

As well as live bacteria (probiotics) which can improve intestinal balance to promote health, dietary carbohydrates may function as prebiotics, beneficially affecting the colonic microflora (Salminen *et al.* 1998b). These dietary carbohydrates include polysaccharides of plant origin (resistant starch, β-glucan, cellulose, inulin), oligosaccharides (fructo-, gluco-, malto-, xylo- and soybean oligosaccharides), and lactose derivatives (Kontula 1999). There have been no reports of the use of EPSs produced by LAB as prebiotics. Although milk fermented with an EPS-producing strain *Lc. lactis* ssp. *cremoris* SBT0495 had cholesterol lowering activity, the mechanism is unknown (Nakajima *et al*. 1992b).

Oda *et al.* (1983) reported an antitumor EPS produced by *Lb. helveticus* ssp. *jugurti.* The antitumor activity of the EPS was tested against ascites Sarcoma-180 by injecting the EPS preparation intraperitoneally. Mice given a 20 mg kg^{-1} dose for nine succesive days had an increased life span value of 144%, and a value of greater than 233% corresponding to a 40 or 80 mg kg^{-1} dose. The authors concluded that the antitumor activity of the EPS might be based on its host-mediated actions. In order to understand the antitumor activity, the effect of the EPSs or the EPS-producing cells on the immune system has been investigated. Forsén *et al*. (1987) showed that cell surface materials, possibly lipoteichoic acids, of *Lc*. *lactis* ssp. *cremoris* T5 produced T-cell mitogenic activity in human lymphocytes.

The slime produced by *Bifidobacterium adolescentis* had immunomodifying effects on mouse splennocytes (Gómez *et al*. 1988). Kitazawa *et al*. (1992) showed that the slime-forming *Lc. lactis* ssp. *cremoris* KVS20 had antitumor activity, and the slime contained strong B-cell dependent mitogenic substances.

3. AIMS OF THE STUDY

One of the aims was to study the antimicrobial compounds produced by dairy lactic acid bacteria, particularly the low-molecular-mass compound inhibitory toward various spoilage and pathogenic bacteria in foods. Another aim was to study the extracellular polysaccharides produced by dairy lactic acid bacteria in view of the role of the exopolysaccharides in the improvement of the texture and consistency of fermented foods. The specific aims of the study were:

1. To separate, purify and identify low-molecular-mass antimicrobial compounds produced by the lactic acid bacterial strains, and to study the antimicrobial properties of these compounds.

2. To isolate exopolysaccharides produced by the lactic acid bacterial strains, to evaluate the primary molecular structures of the exopolysaccharides, and to study the rheological properties of the viscous exopolysaccharides.

4. MATERIALS AND METHODS

4.1. Antimicrobial compounds produced by LAB (I, II)

4.1.1. Bacterial strains and growth conditions

All bacterial strains used in the study of antimicrobial compounds were obtained from Valio Ltd, Research and Development Service, Helsinki, Finland. The bacterial cultures were maintained at -80 °C in glass beads and they were subcultured twice before use. The LAB strains examined for producing antimicrobial compounds were grown in MRS, KCA, and whey permeate or whey media, and incubated at 30 °C or 37 °C (Table 3). Food spoilage bacteria and also LAB strains (Table I/1, Table II/2) were used as indicator organisms for antimicrobial tests.

Table 3. List of lactic acid bacterial strains examined for producing antimicrobial compounds, their growth media and incubation conditions used in this study

Strain	Growth medium	Incubation condition
Lactobacillus sp.		
acidophilus "NCFB" Lb 1748	MRS	30 °C, 25 h
casei C	MRS	30 °C, 25 h
casei ssp. *casei* LC-10	MRS	37 °C, 72 h
casei LC1/6-1	MRS	30 °C, 25 h
casei SHIROTA	MRS	37 °C, 25 h
delbrueckii ssp. *delbrueckii* strain 13S	MRS	37 °C, 25 h
helveticus Äki4	MRS	37 °C, 45 h
lactis KKNO 1134 Lb78	MRS	37 °C, 25 h
lactis ssp. *bulgaricus* KKNO 312 Lb389	MRS	37 °C, 25 h
paracasei ssp. *paracasei* Lb1931	MRS	37 °C, 72 h
reuteri DSM20016	MRS	30 °C, 25 h
rhamnosus GG	MRS	37 °C, 24 h
rhamnosus LC-705	Whey permeate	30 °C, 48 h
Lactococcus lactis ssp. *diacetylactis* EM1*	KCA**	30 °C, 24 h
Pediococcus sp.		
strains VN13, VN18, 435, 4025, 4035	MRS	30 °C, 24 h
Streptococcus thermophilus T101/85*	Whey medium	37 °C, 18 h

* Unpublished.

* * KCA: calcium citrate agar.

4.1.2. Separation and purification of antimicrobial compounds

After the growth of the LAB strains under proper conditions (Table 3), cells in the culture broth were filtered, and the cell-free broth was concentrated 10-fold by lyophilization. The concentrate was then precipitated stepwise by ethanol from 30 to 97.5% with intermediate centrifugation (30 min, 22 000g, 4 °C). The precipitates obtained from each addition of ethanol and/or the final supernatants showing antimicrobial activity were further purified by chromatographic methods.

Gel filtration was performed using a Bio-Rad Econo System (Richmond, CA, USA). The sample (100 mg) was loaded onto a column (75 x 1.5 cm) on Bio-Gel P-2 polyacrylamide gel (M=100-1800, -400 mesh, Bio-Rad) eluted with 0.05 M ammonium acetate (NH_4OAc) at a flow rate of 10 ml h^{-1}, and the eluant was monitored at 280 nm. The active fractions were collected,

lyophilized, and subjected to anion exchange chromatography using a Bio-Rad Econo system with a column (25 x 1.5 cm) on weakly basic Fractogel TSK DEAE-650(S) gel (Merck, Darmstadt, Germany). Elution was carried out at a flow rate of 1.0 ml min^{-1} using a stepwise elution program: fractions 1-30 with water; fractions 31-65 with 0.04 M NH_4OAc adjusted to pH 5.5 with acetic acid (AcOH); fractions 66-90 with 0.5 M NH_4OAc adjusted to pH 5.5 with AcOH. A fraction was collected every four minutes with monitoring at 254 nm.

The active fractions (except fractions containing lactic acid) from anion exchange chromatography were further purified by RP-HPLC using a model 600 E multisolvent delivery system equipped with a Baseline 810 software (Millipore Co., Milford, MA, USA). The mobile phase, 0.02 M NH_4OAc containing 1% AcOH (pH 3.80), was used after filtration through a membrane filter (pore size, 0.2 μm). Elution was performed isocratically from a Spherisorb S5 C8 column (250 x 4.6 mm, Phase Separations Ltd, Chester, England), fitted with a C8 precolumn (Millipore) at a flow rate of 0.75 ml min^{-1}, and at 40 °C for 30 min. A fraction was collected each minute. The absorbance was monitored at a range of wavelengths from 190 to 300 nm at an interval of 5 or 10 nm.

4.1.3. Identification of antimicrobial compounds

^{1}H and ^{13}C NMR measurements were carried out on a Bruker AM 400 WB spectrometer (Karlsruhe, Germany), operating at 400.1 MHz for ^{1}H. Spectra were recorded with sample solutions in H_2O/D_2O (90/10) at ambient temperature and referenced to sodium 3-trimethylsilyl-[2,2,3,3-$^{2}H_4$]propanoate.

The electron impact (EI) and fast atom bombardment (FAB) mass spectra were recorded on a Jeol SX-102 double-focusing spectrometer (Tokyo, Japan).

EI: The sample was injected into the direct probe and the solvent (water) evaporated. The probe was inserted into the ion source (250 °C). The filament was heated at a rate of 16 °C/min up to 300 °C/min, the ionization current being 300 mA. The ionization energy was 70 eV and the accelerating voltage 10 kV. The spectra were recorded over the range 10-500 *m/z*. Calibration was based on PFK (perfluorokerosin, positive ion mode).

FAB: The sample was introduced on the target plate directly into the ion source (40 °C) in a glycerol matrix. The target was bombarded with xenon atoms having a maximum of 6 kV energy. The acceleration voltage of generated ions was 10 kV. The spectra were recorded at a scan range of 0-800 *m/z*. Calibration was based on solid CsI (cesium iodide, positive ion mode).

4.1.4. Antimicrobial assay

The agar diffusion method was performed using a disc test and a spot test. The disc test was performed according to a procedure developed by Pulusani *et al*. (1979) with some modifications: 10 ml of the melted agar medium was seeded with 100 μl of an 18 ± 2 h old broth culture of the test organism in a sterile petri dish. When the soft agar had hardened, an antibiotic test disc (diameter 6 mm, Schleicher & Schuell) was placed on the agar surface, and 22 μl of the sample was spotted onto the disc. After incubation for 20 ± 2 h at the appropriate temperature for each organism tested, the diameter of the inhibition zone around the disc was measured. The spot test was done by spotting the liquid sample (3 μl) directly onto the surface of the solidified, seeded agar medium, and the diameter of the inhibition zone was measured after incubation.

Turbidometric assays were performed using a Bioscreen C automated turbidometer equipped with a Biolink software (Labsystems Co., Helsinki, Finland). The growth of indicator

organisms in broth (300 µl) containing antimicrobial compounds was studied in plates (100 wells). Each well was inoculated with 100 µl broth culture (grown overnight) of the test organism diluted to 10^6 to 10^7 CFU ml^{-1}. The optical density was measured automatically at 30 min-interval, using a wideband filter (405-600 nm), and the plates were shaken at 3 min-interval for 20 s. The growth curves were determined from the turbidity data.

4.2. EPSs produced by LAB (III, IV, V)

4.2.1. Bacterial strains and growth conditions

The LAB strains examined for producing EPSs and their growth conditions are shown in Table 4. The source and methods of maintainance of these strains were the same as described above for the LAB strains examined for producing antimicrobial compounds in this study (4.1.1.)

Table 4. Lactic acid bacterial strains examined for producing exopolysaccharides, their growth media and incubation conditions used in this study

Strain	Growth medium	Incubation condition
Lactobacillus sp.		
fermentum G.1.2.1*	Whey medium	37°C, 18 h
helveticus Äki4	MRS	37°C, 45 h
helveticus Lb161	Skim milk	37°C, 20 h
helveticus K16*	Skim milk	37°C, 24 h
rhamnosus LC705*	Skim milk	30°C, 24 h
rhamnosus GG*	Lactose-hydrolyzed milk	30°C, 20 h
Lactococcus sp.		
lactis ssp. *cremoris* strains ARH 53, ARH 74, ARH 84, ARH 87, B30	Skim milk	25°C, 18-20 h
lactis ssp. *cremoris* SEPH 11*	Skim milk	25°C, 18-20 h
Streptococcus thermophilus THS/41*	Skim milk	37°C, 18 h

* Unpublished.

4.2.2. Isolation of EPSs

For the isolation of the EPS produced by *Lb. helveticus* Äki4 grown in MRS broth, bacterial cells were filtered from the medium, and the cell-free supernatant was concentrated 10-fold by lyophilization. The concentrate was fractionally precipitated with ethanol from 40 to 95% with intermediate centrifugation. The polysaccharide precipitated at 40% ethanol was washed, and dissolved in water. After filtration through a syringe filter (0.8 µ/0.2 µl), it was freeze-dried. The crude polysaccharide (20 mg) was purified by anion-exchange chromatography with a column (25 x 1.5 cm) on Fractogel TSK DEAE-650(S) gel (Merck) using a Bio-Rad Econo system. The column was eluted at about 60 ml h^{-1} first with water for 80 min, and subsequently with 0.06 M NH_4OAc adjusted to pH 5.5 with AcOH for 120 min. A fraction was collected every eight minutes with monitoring at 254 nm, and the presence of sugar was tested with a Molish reagent (Miller and Neuzil 1982).

For the isolation of the EPSs produced by other LAB strains grown in milk or whey medium (Table 4), proteins and cells were initially precipitated by addition of 4% (w/v) TCA (Merck) to the culture, and the mixture was stirred for 2 h. After centrifugation (35 min, 22 000 g, 4 °C), the supernatant was collected and filtered. Cold ethanol was then gradually added to the

cell-free supernatant from one to two, and three volumes of the supernatant with intermediate centrifugation. The EPS precipitated was washed and dissolved in water. The aqueous solutions of EPS were filtered, and then extensively dialyzed against water overnight at 4 °C with two changes of water, and finally lyophilized. The purity of the EPS material was examined by gel filtration using a column (75 x 1.5 cm) of Bio-Gel P-30 polyacrylamide gel (exclusion limit 40 000 daltons, 100-200 mesh). The sample (1 mg) was loaded onto the column and eluted with 0.05 M NH_4OAc with UV monitoring at 280 nm. To check the ionic nature of the EPSs, anion-exchange chromatography of the EPS solutions (~1 mg mL^{-1}) was performed using a column (25 x 1.5 cm) of weakly basic Fractogel TSK DEAE-650(S) gel. Elution was carried out at 1.1 mL min^{-1}; first with water for 2 h, and subsequently with NH_4OAc from 0.1 to 0.5 M using a linear increasing gradient.

4.2.3. Structural elucidation of EPSs

GC analysis of alditol acetates was performed on a HP-5 fused silica column (0.20 mm x 25 m) using a temperature program of 180 °C for 1 min followed by 3 °C min^{-1} to 250 °C. Hydrogen was used as the carrier gas. The column was fitted to a Hewlett-Packard model 5890 series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a flame ionization detector. GLC-MS analysis was performed on a Hewlett-Packard model 5970 mass spectrometer equipped with an HP-5MS fused silica column (0.2 mm x 25 m). A temperature program of 170 °C for 3 min followed by 3 °C min^{-1} to 250 °C was used with helium as the carrier gas.

In sugar analysis, the EPS samples were hydrolyzed with 2 M TFA at 120 °C for 2 h. After reduction with sodium borohydride ($NaBH_4$) and acetylation, the samples were analyzed by GC. The absolute configuration of the sugars present in the EPSs was determined essentially as devised by Leontein *et al*. (1978) but with (+)-2-butanol (Gerwig *et al*. 1978).

Methylation analysis was performed according to Hakomori (1964) using sodium methylsulfinylmethanide and iodomethane in dimethyl sulfoxide. The methylated compounds were recovered by use of Sep-Pak C18 cartridges (Millipore) using the method of Waeghe *et al*. (1983). The purified methylated sample was then hydrolyzed (2 M TFA, 120 °C, 2 h), reduced, and acetylated. The partially methylated alditol acetates were analyzed by GLC-MS.

NMR spectra of solutions in D_2O were recorded at 65 °C and pD 5.5, using a Jeol GSX-270, Jeol Alpha-400, or Varian Inova 600 or 800 MHz instrument. Chemical shifts are reported in ppm relative to sodium 3-trimethyl-(2,2,3,3-$^2H_4$)propanoate (δ_H 0.00) or acetone (δ_c 31.00) as internal references, or dioxan as an external reference. Data processing was performed using standard Jeol software, VNMR software, or Felix 2.3 (Biosym/MSI, San Diego, CA, USA). $^1H,^1H$-COSY, relayed COSY, double-relayed COSY, TOCSY, $^{13}C,^1H$-COSY, gHSQC (Wilker *et al*. 1993) and HMBC (Bax and Summers 1986) experiments were used to assign signals and performed according to standard pulse sequences. For inter-residue correlations, 2D NOESY experiments with mixing times of 300 and 400 ms (III), or 75 and 150 ms (IV), and HMBC experiments with 60 and 90 ms (III), or 45 and 90 ms (IV) delays for the evolution of long-range couplings were used.

4.2.4. Rheological measurements of the EPSs produced by *Lc. lactis* ssp. *cremoris* strains

The viscosities of the dilute solutions of EPS at concentrations of 0.01 up to 0.1 g dL^{-1} were measured at 25 °C with an Ubbelohde capillary viscometer (536 13/Ic, SCHOTT-

GERÄTE, Hofheim, Germany), which allows the determination of the flow times with an accuracy of 0.03 s. The aqueous solutions of EPS with or without the addition of salt were prepared by dissolving a measured amount of EPS in 0.1 M sodium chloride (NaCl) solution or in deionized water. Sample dilution to the various required concentrations of EPS was done directly in the viscometer. After about 5 min for temperature equilibration, flow times were taken, and each flow time was reproduced six times. The reduced viscosity and intrinsic viscosity of the EPS solutions were calculated from the collected data.

The effect of temperature, pH and salts on the rheological behavior of the EPS solutions (1%, w /v) was studied with a Bohlin VOR rheometer (Bohlin Instruments Ltd, England) using concentric cylinders (C14) with a gap of 0.5 mm between the upper and lower geometries. The viscometry measurements were performed at 5, 25, 40 or 60 °C, with increasing shear rates up to 291 s^{-1} in 29 steps. The pH of the EPS solutions was adjusted with lactic acid to 4.0, 5.0 and 6.5. The EPS solutions containing salts were prepared by addition of EPS to 0.1 M NaCl or 0.1 M calcium chloride ($CaCl_2$) solutions. The oscillation measurements were performed for the EPS (1%, w/v) in aqueous and salt solutions, and in skim milk at a frequency sweep from 0.01 to 15 Hz in 19 steps. For every temperature-dependent measurement a thermal equilibration time of about 60 min was used.

5. RESULTS AND DISCUSSION

5.1. Antimicrobial compounds produced by LAB (I, II, unpublished results)

5.1.1. Separation, purification and identification of antimicrobial compounds

After ethanol precipitation of the cell-free cultures of the LAB strains (Table 3), the obtained precipitates and the final supernatants were tested for antimicrobial activity. The supernatants containing antimicrobial activity were subjected to chromatographic separation and purification. The active fractions appeared in a relatively narrow part of the chromatogram on gel-filtration on Bio-Gel P-2 of the supernatants (Fig. 1/I). Anion exchange chromatography of these active fractions (Fig. 2/I) resulted in two different ranges of active fractions (52-54 and 58-63), fractions 58-63 containing lactic acid. Further purification by RP-HPLC of the fractions 52-54 gave rise to one major peak at retention time 4.96 min and two small peaks at 3.65 and 5.78 min. The absorption maximum of the antimicrobial compound was at 215 nm (Fig. 3/I). Fractions of these peaks were collected and tested for antimicrobial activity. Only the fraction of the peak at 4.96 min was found to contain antimicrobial activivity. Identification of this active fraction by both NMR (^{1}H and ^{13}C) and MS (EI and FAB) spectra indicated that the antimicrobial compound was 2-pyrrolidone-5-carboxylic acid (PCA), also known as pyroglutamic acid.

Among the twenty LAB strains (Table 3) examined, thirteen *Lactobacillus* and five *Pediococcus* strains were found to produce PCA under the growth conditions used in this study (Table 5). Four *Lactobacillus* strains produced, in addition to PCA, also HMM antimicrobial compounds, as indicated by the presence of antimicrobial activity in the precipitates obtained from ethanol precipitation. Since these HMM compounds exhibited a rather narrow range of activity (Table 5) they were not subjected to further studies. On the basis of the results of this study, it seems that many LAB strains, particularly *Lactobacillus* strains, are able to produce PCA.

Since LAB produce relatively large amounts of lactic acid, being antimicrobially active, it is important to remove the lactic acid in order to find other antimicrobial compounds, especially those of low molecular mass. In the separation and purification procedures developed in this study, both PCA and lactic acid were present in the culture supernatant obtained from ethanol precipitation, and they also appeared in almost the same range of fractions after gel filtration. However, complete separation of lactic acid was achieved by anion exchange chromatography based on a gel matrix (Fractogel TSK) suitable for separation of biomolecules. Previously, size exclusion HPLC was used to separate lactic acid from LMM antimicrobials, but all the fractions obtained were found to contain antimicrobial activity because the mobile phase (sodium phosphate) used was antimicrobially active (Lortie *et al*. 1993). Niku-Paavola *et al*. (1999) reported the separation of lactic acid by gel filtration on Sephadex G-10 with water as an eluent and found several LMM antimicrobial compounds produced by *Lb. plantarum*. Although there were some reports on the separation and purification of LMM antimicrobial compounds produced by LAB (Pulusani *et al*. 1979, Reddy and Ranganathan 1983, Mehta *et al*. 1984), the techniques for seperation of lactic acid appeared not to be clearly demonstrated. In addition, there were reports (Nielsen *et al*. 1990) on the use of a neutralization technique to eliminate the antimicrobial effect of lactic acid, but lactic acid could not be separated with this technique, and the activity of acidic antimicrobials might be suppressed due to neutralization.

PCA is a natural constituent of foods of plant origin, including vegetables and fruits (Airaudo *et al*. 1987), and fermented soybean and cereal products (Masaaki *et al.* 1992, Syuhei *et al.* 1994). Among LAB strains, only *S. bovis* has previously been shown to produce PCA by conversion of glutamine (Chen and Russel 1989). PCA can also be synthesized by heating

glutamic acid using a dehydration process (Mijin *et al*. 1989). It has been reported that PCA is able to increase cerebral blood flow and decrease the resistance of brain vessels, which result in enhanced brain metabolism, i.e. increased glucose uptake and utilization by cerebral tissues and decreased brain lactate dehydrogenase activity (Mirizoian *et al*. 1994). Other biological functions of PCA are related to its presence as an amino-terminal residue in many biologically significant peptides and proteins, e.g., eisenine, LH-RH (luteinizing hormone releasing hormone), and TRH (thyrotropin releasing hormone) (Pattabhi and Venkatesan 1974, Abraham and Podell 1981, Paul *et al*. 1990).

Table 5. Lactic acid bacterial strains producing antimicrobial compounds and the sensitive strains for the HMM compounds (Table 1 in paper II and unpublished results)

Producing strain	Antimicrobial compound	Sensitive strains for the HMM compounds*
Lactobacillus sp.		
acidophilus "NCFB" Lb1748	PCA	
	HMM compounds*	*Lb. delbrueckii* ssp. *bulgaricus* KKNO 293
		Lb. helveticus 632 KKNO 1129
		L. lactis ssp. *lactis* Lb430 KKNO 1512
		L. lactis ssp. *lactis* KKNO 1189
casei C	PCA	
casei ssp. *casei* LC-10	PCA	
casei LC1/6-1	PCA	
casei SHIROTA	PCA	
delbrueckii ssp. *delbrueckii* strain 13S	PCA	
	HMM compounds*	*Lb. delbrueckii* ssp. *bulgaricus* KKNO 293
helveticus Äki4	PCA	
	HMM compounds*	*Lb. delbrueckii* ssp. *bulgaricus* KKNO 293
lactis KKNO 1134 Lb78	PCA	
lactis ssp. *bulgaricus* KKNO 312 Lb389	PCA	
paracasei ssp. *paracasei* Lb1931	PCA	
reuteri DSM20016	PCA	
	HMM compounds*	*Lc. lactis* ssp. *lactis* Lb430 KKNO 1512
rhamnosus GG	PCA	
rhamnosus LC-705	PCA	
Pediococcus sp.		
strains VN13, VN18, 435, 4025, 4035	PCA	

* Unpublished.

5.1.2. The antimicrobial activity of 2-pyrrolidone-5-carboxylic acid produced by LAB

Although LAB are able to produce a variety of antimicrobial compounds, the present study, for the first time, reports that the production of a certain cyclic amino acid (PCA) is involved in the antimicrobial action of LAB. PCA has been considered, following the identification of reuterin, to be another well identified LMM antimicrobial compound produced by LAB (Ouwehand 1998).

The antimicrobial assay of PCA by agar diffusion method showed that PCA at 2% inhibited *Bacillus subtilis*, *E. coli, Enterobacter, Klebsiella*, and *Pseudomonas* strains. The most sensitive strains *Enterobacter cloacae* 1575, *Pseudomonas fluorescens* KJL G, and *Pseudomonas putida* 1560-2 were inhibited by PCA at 0.5% and 1.0%. Among all bacterial strains tested (Table 1/I and Table 2/II), the Gram-positive strains such as LAB strains, and

several *Listeria* and *Staphylococcus* strains were not inhibited by PCA. An *Enterococcus faecalis* strain was moderately inhibited by 0.5% PCA in BHI broth at 37 °C. It seemed that Gram-negative bacteria were more sensitive to PCA than the Gram-positive ones. This is in agreement with previous studies which showed that organic acids (lactic and acetic acids) were more inhibitory toward Gram-negative bacteria than Gram-positive ones (Doores 1993, Shelef 1994).

PCA was heat stable and the antimicrobial activity did not change after heat treatments (63 °C 30 min, 72 °C 15 s or 121 °C 20 min). Although raising the pH of the PCA in water solutions reduced the antimicrobial activity, and completely destroyed the activity at pH 3.8-4.0 (Table 3/II), the antimicrobial activity of PCA seemed to be not solely due to the pH effect, but other factors may be possibly involved. The turbidometric assay of antimicrobial activity showed that PCA in broths was inhibitory against the test organisms at pH 5.0-5.89 (Table 4/II). At pH values when 1% PCA showed no activity, 2% PCA was still active (Table 3/II). Earlier studies showed that neutralization caused the loss of the antimicrobial activity of *Lb. acidophilus* (Tramer 1966, Shahani *et al*. 1972, Reddy *et al*. 1984), since many inhibitory substances produced by lactic cultures were relative stable in an acidic pH (Mehta *et al*. 1984). In addition, the antimicrobial activity of PCA, like other organic acids, may also be dependent on the undissociated form of the acid.

PCA was found to be less inhibitory than lactic acid. At the same concentrations, lactic acid was more active than PCA against several indicator organisms tested (Fig. 1/II). During the course of this study, we also observed that the production of PCA varied with strains and it was generally small when compared with the amount of lactic acid produced. However, the significance of PCA is that, in addition to its wide spectrum of antimicrobial activity, it is also involved in many important biological functions as discussed above. Among the PCA-producing LAB strains of this study, *Lb. rhmnosus* GG was found to produce relative large amounts of PCA. *Lb. rhamnosus* GG has also been reported to produce a microcin-like LMM antimicrobial compound (Silva *et al*. 1987), and the strain has been commercially applied in the production of probiotic milk products, e.g. Gefilus in Finland (Salminen 1994). Considering the potential applications of PCA, e.g. as PCA-producing starters in food preservation, further studies are needed on the optimal production of PCA by LAB strains, the antimicrobial effects of PCA in foods and the effective PCA concentrations, as well as the sensory quality of foods when PCA is used.

5.2. EPSs produced by LAB (III - V, unpublished results)

Of the thirteen LAB strains (Table 4) examined, except for *Lb. fermentum* G.1.2.1, *Lb. rhamnosus* LC705 and *Lc. lactis* ssp. *cremoris* SEPH 11 which did not produce EPSs, ten strains produced neutral or anionic EPSs of varying amounts when they were grown under the conditions of this study (Table 6).

Previous studies have shown that the production of EPS is growth-associated, and it is influenced by medium compositions, culture temperature and pH (Grobben 1998). Gamar *et al*. (1997) showed that variations in carbon sources in the growth medium resulted in different yields of the EPS produced by *Lb. rhamnosus* strain C83. Addition of glucose or sucrose to the milk medium stimulated the EPS production and modified the sugar composition of the EPS produced by *Lb. casei* ssp. *casei* NCIB 4114 (Cerning *et al*. 1992). Several authors have also reported that more EPSs could be produced by different LAB strains at lower temperatures, or with limited nitrogen sources (Cerning *et al*. 1992, van den Berg *et al*. 1995, Marshall *et al*. 1995). Although bacteria grow well, and more cells may be obtained under optimized growth conditions, unfavourable culture conditions may stimulate EPS production by the cells as a form of bacterial

self-protection. Therefore, these factors discussed above need to be considered for optimizing EPS production by the LAB strains in this study.

Table 6. Lactic acid bacterial strains producing exopolysaccharides, the yields and charge

Producing strain**	EPS Yield (mg L^{-1} culture)	EPS charge	Reference
Lactobacillus sp.			
helveticus Äki4 (=2091)	~500	neutral	III
helveticus Lb161	260*	neutral	IV
helveticus K16	110	neutral	unpublished
rhamnosus GG	80	neutral	unpublished
Lactococcus lactis ssp. *cremoris*			
strain ARH53	263	anionic	V
strain ARH74	222	anionic	V
strain ARH84	215	anionic	V
strain ARH87	225	anionic	V
strain B30	164	anionic	V
Streptococcus thermophilus THS/41*	60	neutral	unpublished

* Unpublished.

* * The growth conditions of the LAB strains are shown in Table 4.

5.2.1. EPSs produced by *Lb. helveticus* strains

As shown in Table 6, *Lb. helveticus* Äki4 grown in MRS broth, and strains Lb161 and K16 grown in skim milk were found to produce EPSs. Viscometric measurements by Ubbelohde-type capillary viscometer showed that at 0.5% (w/v) EPS in aqueous solutions, the EPSs of strains Lb161 and K16 were viscous, but the EPS of strain Äki was not viscous. The primary molecular structures of the EPSs produced by strains Äki4 and Lb161 have been studied by sugar and methylation analyses, and 1D and 2D NMR spectroscopy (III, IV).

5.2.1.1. Structural elucidation of the EPSs produced by *Lb. helveticus* Äki4 (III) and Lb161 (IV)

The EPS produced by strain Äki4 (in paper III designated as strain 2091) was shown to be composed of a hexasaccharide repeating unit containing one terminal β-D-galactose, one 4-substituted α-D-galactose, one 6-substituted β-D-galactose, one 4-substituted β-D-glucose, one 3,6-substituted β-D-galactose and one 6-substituted β-D-glucose residue. The assignment of the spin systems for the six sugar residues was performed using 2D homo- and heteronuclear techniques (Table 1/III). Each spin system with a specific sugar residue and substitution pattern was identified from the $J_{H,H}$ values, indicating the anomeric configuration, and from the ^{1}H and ^{13}C NMR chemical shifts. The sequence of the sugar residues was determined using 2D NOESY and HMBC experiments. The structure of the repeating unit of the EPS is as follows:

→6)-β-D-Gal*p*-(1→4)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-β-D-Glc*p*-(1→

6
↑
1
β-D-Gal*p*

The EPS produced by strain Lb161 was shown to be composed of a heptasaccharide repeating unit containing two terminal β-D-glucose, one 2,3-substituted α-D-glucose, one 3-substituted α-D-galactose, one 4-substituted α-D-glucose, one 3,4-substituted β-D-galactose and one 3-substituted β-D-glucose residue. The assignments of the spin systems for the seven sugar residues were performed using 2D homo- and heteronuclear techniques (Table 1/IV)). Each spin system with a specific sugar residue and substitution pattern was identified on the basis of the $J_{H,H}$ values and the ^{1}H and ^{13}C NMR chemical shifts. The sequence of the repeating unit was established using 2D NOESY and HMBC experiments with the following structure:

→4)-α-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→3)-α-D-Gal*p*-(1→2)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→

3 3
↑ ↑
1 1
β-D-Glc*p* β-D-Glc*p*

5.2.1.2. Structural variations among the EPSs produced by *Lb. helveticus* strains

As shown above, the repeating units of the EPSs of *Lb helveticus* Äki4 and Lb161 are a hexamer and a heptamer made up of a main chain branched by one and two side chains, respectively. Structural studies on the EPS of strain K16 showed that the repeating unit of the EPS was composed of six sugars: one terminal D-galactose, one terminal D-glucose, one 4-substituted D-galactose, one 4-substituted D-glucose, one 2,4-substituted D-glucose and one 4,6-substituted D-glucose (unpublished data).

Comparing with the so far published structures of the EPSs of other four *Lb. helveticus* strains (Fig. 1 in the literature review), the common structural feature of the EPSs produced by all these *Lb. helveticus* strains is that the EPSs contain either a hexa- or heptasaccharide repeating unit of D-galactose and D-glucose. The EPS produced by *Lb. helveticus* strain TY1-2 (Fig. 1) contains *N*-acetyl-D-glucosamine in addition to D-galactose and D-glucose (Yamamoto *et al*. 1994). Rhamnose and other monosaccharides which have been found in the EPSs produced by many LAB strains are not present in the EPSs of the *Lb. helveticus* strains. Since the rheological properties of polysaccharides are closely related to their primary molecular structures and three-dimensional structures, it would be of interest to study the relation between structures and functional properties of the EPSs produced by LAB strains within the same species. The difference in the viscosities of the EPSs of the *Lb. helveticus* strains of this study is probably due to their different primary molecular structures (linkage and degree of branching), molecular masses, three-dimensional structures and chain-chain interactions of the polysaccharides in solutions.

5.2.2. EPSs produced by *Lc. lactis* ssp. *cremoris* strains and their rheological characterization (V)

The growth of *Lc. lactis* ssp. *cremoris* strains (ARH53, ARH74, ARH84, ARH87, B40) in skim milk at 25 °C for 18-20 h resulted in very viscous cultures as compared to the *Lb. helveticus* strains of this study. The EPSs (164-263 mg L^{-1}) produced by these 'viili' strains were separated and purified from the cultures by treatment with 4% TCA and ethanol precipitation (Table 1/V). The EPSs were shown to be anionic in nature by anion exchange chromatography, and they contained the same monosaccharides rhamnose, glucose and galactose in similar molar ratios (Table 1/V). The combined evidence from sugar analysis and NMR spectroscopy (Fig.1/V) indicates that the primary molecular structures of these EPSs are identical or closely related to that from *Lc. lactic* ssp. *cremoris* SBT 0495 (Nakajima *et al*. 1992a). The underestimation of the ratio of galactose (Table 1/V) resulted from incomplete release by acid hydrolysis. The galactose residues were involved in the phophodiester linkage in the polysaccharide and it was hard to release them.

In dilute aqueous solutions (up to 0.1 g dL^{-1}), the EPSs produced by *Lc. lactis* ssp. *cremoris* strains exhibited a polyelectrolyte effect (Fig. 3/V). At very low concentrations (0.02 g dL^{-1}), an increase in reduced viscosity with a decrease in concentration was observed. In the presence of 0.1 M NaCl, the reduced viscosities of all the EPS solutions were significantly lowered, and the polyelectrolyte effect disappeared as indicated by the straight lines of the Huggins plot. The intrinsic viscosities of the EPSs were therefore obtained by a linear extrapolation of the plots to zero EPS concentration (Table 1/V). Since the EPS produced by strain ARH53 gave the highest intrinsic viscosity (19.62 dL g^{-1}), this EPS might possess a relatively large molecular size than the other *Lc. lactis* ssp. *cremoris* strains studied.

At a higher concentration (1%, w/v), the viscosities of the aqueous solutions of EPS produced by strain ARH53 were temperature, pH and salt dependent. At the same pH (4.0, 5.0 or 6.5), increasing temperature from 5 °C to 60 °C caused a decrease in viscosity. At higher temperatures (40 °C and 60 °C), the viscosity was clearly lowered in the order of pH 6.5 > pH 5.0 > pH 4.0 (Figs. 4C and 4D/V). The higher viscosity of EPS in 0.1 M $CaCl_2$ solutions than that in 0.1 M NaCl solutions (Fig. 5A/V) is due to different interactions between cations and polyelectrolyte chains, Ca^{2+} being more effective than Na^{+} in this respect (Parker *et al*. 1996). Changing Na^{+} into Ca^{2+} ion was observed also to increase the storage modulus of the sample (Fig. 5B/V). Samain *et al*. (1997) compared the effect of salts on the viscosity of a gel-forming EPS produced by *Alteromonas sp.* strain 1644 and showed that the divalent magnesium cation was more effective than the monovalent sodium cation.

The aqueous solutions of the EPS (1%, w/v) of strain ARH53 behave viscoelastically. As shown in the oscillation measurements (Fig. 7/V) at 5, 25 and 40 °C, the EPS solutions behaved as a viscoelastic fluid at lower frequencies with G" > G', and showed a predominant elastic character (G' > G") at higher frequencies. The drastic decrease in viscosity of the EPS aqueous solution with increasing shear rate (Fig. 6/V) also clearly shows the non-Newtonian behavior (shear-thinning) of the solution.

In skim milk to which the EPS of strain ARH53 was added, the viscosity was much higher as compared with the EPS in aqueous solutions at 5, 25 and 40 °C (Fig. 6/V). At shear rates lower than 9.21 s^{-1}, an upward shift in viscosity was observed at 40 °C. The viscosity was higher at 40 °C than at 25 °C, and even higher than at 5 °C at shear rates lower than 0.37 s^{-1}. In the oscillation measurements, at 5 °C there is a moderate frequency dependence of G' with a crossover of G' and G" at the lower end of the frequency range, suggesting the formation of a weak gel (Fig. 7A/V). At 25 °C the system exhibited a viscoelastic behavior with a crossover of

G' and G" at about 0.8 Hz (Fig. 7B/V). Increasing temperature to 40 °C led to the formation of a strong gel, as indicated by G' » G", and the parallel curves of G' and G" with only a slight frequency dependence, and the value of G' being 198 Pascals at 15 Hz (Fig. 7C/V). The interactions between EPS and casein micelles of skim milk were possibly involved in the gelation. Hess *et al*. (1997) proposed that in yoghurt the interactions between EPS and casein micelles are stronger than those between the casein micelles. Intercalation of the EPSs into the casein matrix was also involved in the improvement of the texture of quarg using ropy cultures (Sebastiani *et al*. 1997). It seemed that the interactions between the EPS and casein micelles increased at higher temperatures as observed in this study.

The production of EPSs by *Lc. lactic* ssp. *cremoris* strains has been reported earlier (see 2.4.2.2.). However, the rheological properties of these EPSs were poorly understood. Recently, Oba *et al*. (1999) reported the viscoelastic properties of the EPS produced by strain SBT 0495. It is of interest that the rheological behavior of this EPS in aqueous solutions, e.g. polyelectrolyte effects and viscoelasticity, is similar to that of the EPS of strain ARH53. This is propably due to their similar or identical structures as shown in this study. The similarity in rheological behavior was also noticed among the EPSs produced by the five 'viili' strains of this study. The difference in viscosity of the EPSs in dilute, as well as in concentrated solutions (data not shown) was possibly due to their different molecular masses.

It has previously been known that slime production is an important factor contributing to the special slimy characteristic of Finnish fermented milk 'viili'. The increase in viscosity and gelling at 40 °C resulted from the addition of the EPS produced by *Lc. lactis* ssp. *cremoris* demonstrated in this study is of interest with respect to the possible use of the EPS to improve the rheological properties of milk products, for instance, yoghurts with fermentation at about 42 °C. Considering the potential applications of the EPSs, future work on the optimization of the EPS production and further physical characterization is required.

6. SUMMARY AND CONCLUSIONS

Lactic acid bacteria are able to produce a large variety of compounds which give fermented foods their characteristic flavor and color, and also impart improved safety and rheology to the foods. In this study, the production of antimicrobial compounds and extracellular polysaccharides by LAB strains obtained from the dairy industry has been investigated in order to find the LAB strains with potential applications in foods.

Studies on the twenty dairy LAB strains from *Lactobacillus* (13), *Lactococcus* (1), *Pediococcus* (5) and *Streptococcus* (1) showed that eighteen strains produced a LMM antimicrobial compound, 2-pyrrolidone-5-carboxylic acid (PCA). Separation and purification of PCA from the growth media were achieved by ethanol precipitation and chromatographic methods. The technique of anion exchange chromatography developed in this study was essential for effective separation of PCA from lactic acid, facilitating the identification of PCA by NMR and mass spectrometry. The chromatographic procedure based on this technique can be used for separation and purification of other LMM antimicrobial compounds. To our knowledge, this is the first report of a cyclic amino acid, PCA, which is involved in the antimicrobial action of LAB. PCA can be considered as a well identified LMM antimicrobial compound following the identification of reuterin produced by *Lb.reuteri* (Paper I).

PCA was shown to be inhibitory toward many food-borne spoilage bacteria such as *Bacillus subtilis*, *Enterobacter*, *E. coli*, *Klebsiella* and *Pseudomonas*. In antimicrobial tests against different organisms, the Gram-negative spoilage bacteria were found to be more sensitive to PCA than the Gram-positive ones. Heat treatment of PCA did not change its antimicrobial activity. Although PCA was active under acidic conditions, it appeared that the activity was not solely due to the pH effect. Regarding the mechanism of antimicrobial action of PCA, more study is needed on the mode of action of PCA on sensitive bacterial cells and the MIC value, as well as the biosynthesis of PCA in different species of LAB. In addition, further investigation on a wide range of LAB strains is needed in order to find out whether production of PCA is a common characteristic of LAB (Paper II).

During the course of the studies on the antimicrobial compounds, we gradually precipitated HMM antimicrobial compounds from culture supernatants by ethanol, while at the same time following how polysaccharides were precipitated. The first EPS, which was precipitated at 40-60% ethanol, was found to be produced by *Lb. helveticus* Äki4 grown in MRS broth. NMR spectroscopic studies of the EPS showed that it was composed of a hexasaccharide repeating unit of D-galactose and D-glucose in a molar ratio of 2:1, and the main chain was branched with a side chain of D-galactose. Although there were relatively large amounts of the EPS (~500 mg L^{-1}) produced by *Lb. helveticus* Äki4, this EPS was found to be not viscous (Paper III).

In the screening study of LAB strains producing viscous EPSs, another *Lb. helveticus* strain, strain Lb161 grown in skim milk was found to produce a viscous EPS. The EPS was isolated by the treatment of the growth medium with TCA to precipitate the proteins first, and subsequently by ethanol precipitation to obtain the polysaccharide. The primary molecular structure of the EPS has been elucidated by NMR spectroscopy. The EPS was shown to consist of a heptasaccharide repeating unit of D-galactose and D-glucose in a molar ratio 2:5 with two side chains, each consisting of a D-glucose residue (Paper IV).

Further studies on slime-forming *Lc. lactis* ssp. *cremoris* strains showed that growth of the strains ARH53, ARH74, ARH84, ARH87 or B30 in skim milk resulted in very slimy cultures. By using basically the same methods as for the EPS of *Lb. helveticus* Lb161, polysacharides of varying amounts were isolated from these cultures. Sugar analysis and NMR spectroscopy

showed that the EPSs had a rather similar or probably identical structure to the one reported earlier. Rheological studies showed that the EPSs in dilute aqueous solutions behaved as polyelectrolytes, and the EPS of strain ARH53 gave the highest intrinsic viscosity. Further characterization of the EPS of strain ARH53 showed that the viscosity of the EPS in concentrated solutions was dependent on the temperature, pH and ionic strength of the solutions. In skim milk, addition of the EPS of strain ARH53 resulted in a clear increase in viscosity and a gel was formed at 40 °C (Paper V).

In our future studies of EPSs, we have planned to continue the study of the EPSs produced by LAB, as well as bifidobacterial strains of food origins, and strains from the human intestine, aiming at applications in food, functional food or clinical products. Although the present study provides data on the structures and rheological properties of EPSs, further studies are needed in order to understand the structure-function relations of the EPSs, the interaction of polysaccharides and proteins (e.g. casein), and the roles of EPSs in their adhesion interaction with EPS-producing strains in the human intestine.

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