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Review

Antifungal lactic acid bacteria as biopreservatives

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Food-borne fungi, both yeasts and moulds, cause serious spoilage of stored food. Moulds may also produce health-damaging mycotoxins, *e.g.* aflatoxins, trichothecenes, fumonisin, ochratoxin A and patulin. Consumer demands for minimally processed foods and reduced use of chemical preservatives have stimulated research on antifungal lactic acid bacteria as biopreservatives. Recently, a number of antifungal metabolites, *e.g.* cyclic dipeptides, phenyllactic acid, proteinaceous compounds, and 3-hydroxylated fatty acids have been isolated from lactic acid bacteria. This review summarizes these findings and suggests potential applications of antifungal lactic acid bacteria in the preservation of food and feeds.

Introduction

Lactic acid bacteria (LAB) are found in many nutrient rich environments and occur naturally in various food products such as dairy and meat products, and vegetables (Carr, Chill, & Maida, 2002). They have traditionally been used as natural biopreservatives of food and feed. Biopreservation refers to extended shelf life and enhanced safety of foods obtained by using the natural or added microflora and their antimicrobial products. The use of fermentation processes has increased during the centuries and now includes many different kinds of food and animal feeds (Ross, Morgan, & Hill, 2002).

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Fungal spoilage of food and feed

Filamentous moulds and yeasts are common spoilage organisms of food products, *e.g.* fermented milk products, cheese, bread, as well as stored crops and feed such as hay and silage (Bonestroo, Dewit, Kusters, & Rombouts, 1993; Bullerman, 1977; Filtenborg, Frisvad, & Thrane, 1996; Moon, 1983). It is estimated that between 5 and 10% of the world's food production is lost due to fungal deterioration (Pitt & Hocking, 1999). In Western Europe, mould spoilage of bread alone is estimated to cause annual economical losses of more than £200 million (Legan, 1993).

Penicillium and *Aspergillus* species are reported as spoilage organisms from a wide range of food and feeds and *Fusarium* species are often found on cereal grains, where they might produce a number of mycotoxins (Filtenborg *et al*., 1996; Samson, Hoekstra, Frisvad, & Filtenborg, 2000). Different *Penicillium* species frequently occur in foods stored under cold conditions, and *P. roqueforti* and *P. commune* commonly spoil hard cheese (Filtenborg *et al*., 1996; Pitt & Hocking, 1999). Yeasts such as *Candida parapsilosis*, *Rhodotorula mucilaginosa*, *Kluyveromyces marxianus* and *Debaromyces hansenii* are common spoilage organisms of yoghurt and other fermented dairy products (Loureiro & Querol, 1999; Pitt & Hocking, 1999).

Traditional control of spoilage fungi

Several techniques are used for the preservation of food and feeds. Drying, freeze-drying, cold storage, modified atmosphere storage, and heat treatments are all means of physical methods of food preservation (Farkas, 2001). Several chemical additives also function as preservatives, even though the exact mechanisms or targets often are not known (Davidson, 2001). The organic acids acetic, lactic, propionic, sorbic and benzoic acids are used as food preservatives (Brul & Coote, 1999). Both sorbic and benzoic acid have a broad spectrum of activity (Davidson, 2001; Nielsen & de Boer, 2000). Benzoic acid and sodium benzoate are used primarily as antifungal agents (Davidson, 2001). The antibiotic natamycin, produced by *Streptomyces natalensis*, is effective against yeasts and moulds and a common preservative on surfaces of hard cheese (Davidson, 2001).

Increasing number of microbial species are becoming resistant to antibiotics. Fungi are no exception and both fungal human pathogens and spoilage moulds in food

and feed systems are becoming resistant. Furthermore, yeasts and moulds are not only becoming resistant to antibiotics, but also to preservatives such as sorbic and benzoic acids, as well as chemical treatment with cleaning compounds (Brul & Coote, 1999; Loureiro, 2000; Sanglard, 2002; Viljoen, 2001). A number of *Penicillium*, *Saccharomyces* and *Zygosaccharomyces* species can grow in the presence of potassium sorbate (Davidson, 2001), and the number of mould species able to degrade sorbate is increasing (Nielsen & de Boer, 2000). In addition, *P. roqueforti* isolates have been found to be resistant to benzoate (Nielsen & de Boer, 2000).

Natamycin-resistant strains of the mould *Penicillium discolor*, a species that causes spoilage of hard cheese, have been found (Cabo, Braber, & Koenraad, 2002; Filtenborg *et al*., 1996; Nielsen & de Boer, 2000). Yeasts like *D. hansenii*, *Candida versatilis* and *Torulaspora delbrueckii*, have also shown strong resistance to chemical sanitizers and cleaning compounds in dairy environments (Viljoen, 2001). There is a great risk that the resistance phenomenon will increase in the future due to the frequent use of antibiotics and preservatives.

The general public demands a reduced use of chemical preservatives or additives in food or feed (Brul & Coote, 1999). Instead, consumers require high quality, preservative free, safe but mildly processed food with extended shelf life. In addition, present legislation may restrict the use of some currently accepted preservatives in different foods (Brul & Coote, 1999).

LAB as biopreservatives

LAB occur naturally in foods or are added as pure cultures to various food products. They are considered to be harmless or even to improve human and animal health (probiotics). LAB have a GRAS status (generally recognized as safe) and it has been estimated that 25% of the European diet and 60% of the diet in many developing countries consists of fermented foods (Stiles, 1996). LAB are well known as starter cultures in the manufacture of dairy products such as acidophilus milk, yoghurt, buttermilk, cottage cheeses, hard cheeses and soft cheeses among others (Carr *et al*., 2002). The coexistence of LAB and fungi is also essential for the success of several biotechnological applications, *e.g.* sourdough bread making, where the ratio of LAB/yeast is generally 100:1 (Gobbetti, 1998). The ancient traditions of using LAB in food and feed, combined with recent knowledge on positive health effects caused by ingestion of probiotic LAB, suggests them as promising alternatives to chemical preservatives.

Antifungal activities of LAB

LAB produce a variety of antimicrobial compounds, *e.g.* the pH-reducing fermentation products lactic and acetic acids, as well as hydrogen peroxide, formic acid, propionic acid, and diacetyl (Lindgren & Dobrogosz, 1990). The precise mechanism of antimicrobial action is difficult to elucidate due to complex and commonly synergistic interactions between different compounds (Corsetti, Gobbetti, Rossi, & Daminani, 1998; Niku-Paavola, Laitila, Mattila-Sandholm, & Haikara, 1999). Research has mainly been directed towards identifying different antimicrobial substances, primarily antibacterial, in simple *in vitro* systems, but little is known about the overall mechanisms of complex preservation systems within food and feed environments (Earnshaw, 1992). Studies on the effect of LAB on fungi are complicated by the sensitivity of most fungi to the normal fermentation products lactic and acetic acids (Bonestroo *et al*., 1993; Lindgren & Dobrogosz, 1990; Piard & Desmazeaud, 1992).

The number of published studies on antifungal LAB is still low and the majority only describe the inhibitory activity of LAB. Until now very few studies have reported on characterization of compounds or mechanisms (Table 1).

Fungal inhibitory metabolites

Organic acids

Lactic acid is the major LAB metabolite, causing pH reductions that inhibit many microorganisms (Eklund, 1989). The undissociated, more hydrophobic form of the acid diffuses over the cell membrane and dissociates inside the cell, releasing H^+-ions that acidify the cytoplasm (Axelsson, 1990; Piard & Desmazeaud, 1991). In addition to the pH effect, the undissociated acid collapses the electrochemical proton gradient, causing bacteriostasis and finally death of susceptible bacteria (Eklund, 1989).

Heterofermentative LAB produce acetic acid in the presence of external electron acceptors in relatively high amounts, whereas propionic acid is only produced in trace amounts. Both acids have higher pK_a values than lactic acid and therefore have a higher proportion of undissociated acid at a certain pH. Similar to lactic acid, acetic and propionic acids interact with cell membranes to neutralize the electrochemical proton gradient, but the effect of acetic and propionic acid is often dependent on the decrease in pH caused by lactic acid (Eklund, 1989; Freese, Sheu, & Galliers, 1973). Propionic acid reduces fungal growth, especially at lower pH (Woolford, 1984a), and affect fungal membranes at pH values below 4.5 (Hunter & Segel, 1973). Propionic and acetic acid also inhibit amino acid uptake (Eklund, 1989; Freese *et al*., 1973). Salts of propionic acid, such as sodium propionate and ammonium propionate show a similar effect against yeast and filamentous moulds at low pH (Woolford, 1984b). Moon (1983) found that mixtures of lactic, acetic and propionic acid inhibited yeast species that normally grow well at relatively high concentrations (100 mM) of the individual acids, except for propionic acid.

The lactic acid produced during LAB growth and the sodium acetate found in the MRS substrate (de Man, Rogosa, & Sharpe, 1960), a standard growth medium for LAB, can have synergistic antifungal effects (Cabo *et al*., 2002; Stiles, Penkar, Plockova, Chumchalova, & Bullerman, 2002). The sodium acetate in MRS might also

Table 1. Compilation of publications reporting antifungal activity of lactic acid bacteria

LAB isolate[a]	Activity spectrum	Compound(s)	Reference
Lactococcus lactis C10	*Aspergillus parasiticus*	ND	Wiseman and Marth (1981)
Lactobacillus casei ATCC 393	*Aspergillus parasiticus*	ND	El-Gendy and Marth (1981)
L. lactis	*Aspergillus flavus*	ND	Coallier-Ascah and Idziak (1985)
L. casei var. *rhamnosus*	Broad spectrum	King, Fowler, and Vandenbergh (1986)	King, Fowler, and Vandenbergh (1986)
L. casei var. *rhamnosus*	Broad spectrum	Vandenbergh and King (1988)	Vandenbergh and King (1988)
Lactobacillus reuteri	Broad spectrum	3-HPA (reuterin)	Chung et al. (1989), Talarico et al. (1988)
Lactobacillus plantarum	Unspecified spoilage mould	ND	Hill (1989)
L. lactis subsp. *diacetylactis* DRC1	*Aspergillus fumigatus*, *Aspergillus parasiticus*, *Rhizopus stolonifer*	Possibly proteinaceous	Batish et al. (1989)
Pediococcus acidilactici	*Saccharomyces cerevesiae*	Possibly proteinaceous	Vandenbergh and Kanka (1989)
Lactobacillus acidophilus R	*Aspergillus fumigatus*	Batish, Lal, and Grover (1990)	Batish, Lal, and Grover (1990)
Lactococcus lactis	*Aspergillus parasiticus*	ND	Luchese and Harrigan (1990)
L. casei subsp. *rhamnosus*, *L. plantarum*, *Leuconocstoc mesenteroides*	*Penicillium* spp., *Aspergillus* spp.	ND	Suzuki, Nomura, and Morichi (1991)
L. plantarum	*Saccharomyces cerevisiae*	ND	Makanjuola, Thymon, and Springham (1992)
L. casei subsp. *Rhamnosus* LC-705	*Candida lusitaniae*, *Aspergillus niger*, *Fusarium* spp., *Penicillium* spp., *Cladosporium* spp.	ND	Mäyrä-Mäkinen, Kristianinkatu, and Suomalainen (1994)
L. lactis subsp. *lactis* CHD 28.3	*Aspergillus flavus*, *A. parasiticus*, *Fusarium* spp.	Possibly proteinaceous	Roy et al. (1996)
L. casei	*Penicillium* spp.	Possibly proteinaceous	Gourama (1997)
Lactobacillus casei subsp. *pseudoplantarum*	*Aspergillus flavus*	Possibly proteinaceous, < 1 kDa	Gourama and Bullerman (1995, 1997)
Lactobacillus sanfrancisensis CB1	*Fusarium* spp., *Penicillium* spp., *Aspergillus* spp., *Monilia* spp.	Caproic acid, propionic acid, butyric acid, valeric acid	Corsetti et al. (1998)
L. plantarum VTT E78076	*Fusarium avenaceum*	Benzoic acid, methylhydantoin, mevalonolactone, cyclo(Gly-L-Leu),	Niku-Paavola et al. (1999)
Lactobacillus pentosus	*Candida albicans*	Pentocin TV35b	Okkers et al. (1999)
L. casei, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. lactis* subsp. *cremoris*	*Penicillium expansum*	ND	Florianowicz (2001)
L. plantarum	Broad spectrum	Phenyllactic acid, 4-hydroxyphenyllactic acid	Lavermicocca et al. (2000)
L. rhamnosus	*Penicillium* spp., *Aspergillus* spp., *Fusarium* spp., *Alternaria* spp.	Sodium acetate[b]	Stiles et al. (2002)
L. plantarum MiLAB 393	Broad spectrum	3-Phenyllactic acid, cyclo(Phe-Pro), cyclo(Phe-OH-Pro)	Ström et al. (2002)
Lactobacillus coryniformis Si3	Broad spectrum	Peptide, phenyllactic acid, cyclo(Phe-Pro), cyclo(Phe-OH-Pro), reuterin	Magnusson (2003), Magnusson and Schnürer (2001), Magnusson et al. (2003)
L. plantarum MiLAB14	Broad spectrum	Hydroxy fatty acids, phenyllactic acid, cyclo(Phe-Pro), cyclo(Phe-OH-Pro),	Magnusson et al. (2003), Sjögren et al. (2003), unpublished results
Pediococcus pentosaceus MiLAB 24	Broad spectrum	Cyclo(phe-OH-Pro)	Unpublished results Atanassova et al. (2003)
Lactobacillus paracasei subsp. *paracasei* M3	*Candida* spp., *Saccharomyces cerevisiae*	proteinaceous	

ND: not determined.
[a] Some species may have received new names through taxonomic revisions.
[b] Sodium acetate from the MRS substrate was involved in the inhibitory action of lactic acid bacteria towards several moulds; the additional effect of other compounds was not determined.

act in synergy with additional antifungal compounds produced by LAB (Stiles *et al.*, 2002). We have observed similar effects (unpublished), but also found that non-inhibiting LAB can produce more lactic acid than strains with high antifungal activity (Magnusson, Ström, Roos, Sjögren, & Schnürer, 2003). Anyhow, the inhibitory effects of organic acids such as lactic, acetic and propionic acid will continue to complicate studies on antimicrobial effects of

LAB, unless rigorous further purification and characterization of substances is applied (Magnusson, 2003; Magnusson & Schnürer, 2001; Magnusson *et al*., 2003; Sjögren, Magnusson, Broberg, Schnürer, & Kenne, 2003).

Other end products

Most LAB have flavoprotein oxidases, enabling them to produce hydrogen peroxide (H_2O_2) in the presence of oxygen. Hydrogen peroxide accumulates in the environment since LAB do not produce catalase (Condon, 1987). The antimicrobial effect of hydrogen peroxide is well documented (Davidson, Post, Branen, & McCurdy, 1983) and attributed to a strong oxidizing effect on the bacterial cell, and to the destruction of basic molecular structures of cellular proteins. The antimicrobial effect of hydrogen peroxide at non-inhibitory concentrations is potentiated by lactoperoxidase and thiocyanate present in milk and saliva (Condon, 1987). The lactoperoxidase–thiocyanate–peroxide system involves the reaction of hydrogen peroxide and thiocyanate through catalysis by lactoperoxidase. Hypothiocyanate and other intermediary products then inhibit other microorganisms. Lactoperoxidase and thiocyanate are present in milk, and when some LAB are grown in milk or milk products, the third needed component, hydrogen peroxide, is added (Björck, 1978; Björck, Rosen, Marshall, & Reiter, 1975). Fitzsimmons and Berry (1994) reported the inhibitory effect of this system against the yeast *Candida albicans*. Rodríguez, Martinez, Suarez, Martinez, and Hernandez (1997) suggested that MRS should be used as substrate when screening for antimicrobial substances other than hydrogen peroxide. They found that hydrogen peroxide is rapidly degraded in MRS, probably due to catalase activity of the yeast extract.

Diacetyl (2,3-butanedione), the characteristic aroma compound of butter, has antimicrobial effects at low pH (Jay, 1982) and is produced by strains of some genera of LAB during citrate fermentation (Earnshaw, 1992). However, the amounts of diacetyl needed to exert antimicrobial activity (close to 200 mM) dramatically alters both the taste and aroma of the product (Piard & Desmazeaud, 1991).

Proteinaceous compounds

Ribosomally synthesized antimicrobial peptides are found in mammals, birds, amphibians, insects, plants and microorganisms. They generally have a hydrophobic and a hydrophilic end, a size of 20–50 amino acids, and cationic properties (Hildeng-Hauge, 1998; Nissen-Meyer & Nes, 1997). Many LAB produce bacteriocins, antibacterial, ribosomally synthesized peptides or proteins (Nes *et al*., 1996). These are generally only active against closely related bacterial species and there is little evidence that bacteriocins should have any effect on fungal growth.

In contrast with the vast literature on bacteriocins, only few reports exist on antifungal peptides of LAB. Several authors have reported that the antifungal activity of LAB is lost after treatment with proteolytic enzymes. Batish, Grover, and Lal (1989) suggested that the antifungal substance produced by a LAB isolate was of proteinaceous nature since activity disappeared with proteinase treatment. Roy, Batish, Grover, and Neelakantan (1996) isolated a *Lactococcus lactis* subsp. *lactis* with antagonistic activity against several filamentous fungi, that was lost after enzymatic treatment with chymotrypsin, trypsin and pronase E. Gourama (1997) found that the inhibitory effect of a *Lactobacillus casei* strain against two *Penicillium* species was slightly reduced by treatment with trypsin and pepsin. Gourama and Bullerman (1995, 1997) showed that a commercially available silage inoculant with a combination of *Lactobacillus* species (*L. plantarum*, *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus*) had antifungal and antiaflatoxin activity against *A. flavus*. When investigating the mixed culture they discovered that a *L. casei* subsp. *pseudoplantarum* (not an intended starter component) was the active microorganism. The inhibitory activity was sensitive to treatments with trypsin and α-chymotrypsin, and it was concluded that the activity was due to a small peptide (<1 kDa). The antiaflatoxigenic properties of LAB could also depend on adherence of fungal toxins to cells of LAB (El-Nezami, Polychronaki, Salminen, & Mykkanen, 2002; Haskard, El-Nezami, Kankaanpaa, Salminen, & Ahokas, 2001).

Only three publications report the purification of antifungal proteinaceous compounds from LAB. Okkers *et al*. (1999) characterized a medium length peptide, TV35b, from *Lactobacillus pentosus* that induced formation of pseudohyphae and had fungistatic effects against *C. albicans*. The peptide was not tested for activity against moulds.

A proteinaceous compound from *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 had antifungal effect against several moulds and yeasts (Magnusson & Schnürer, 2001). The peptide was small (approximately 3 kDa), heat stable, active in the pH range 3–6 and totally inactivated by proteinase K (Magnusson & Schnürer, 2001). Similar characteristics are found among the heat stable, unmodified bacteriocins of subclass II (Nes & Holo, 2000). The production of the antifungal peptide followed the same kinetics as the bacteriocins amylovorin L471 from *Lactobacillus amylovorus* (Callewaert *et al*., 1999), Lactocin S from *Lactobacillus sakei* (Mørtvedt-Abildgaard *et al*., 1995) and the fungistatic peptide TV35b from *L. pentosus* (Okkers *et al*., 1999; Magnusson & Schnürer, 2001).

When a liquid culture of *L. coryniformis* strain Si3 was supplemented with either ethanol, formic or acetic acid the total amount of the antifungal peptide increased (Magnusson & Schnürer, 2001). Data suggested that the antifungal peptide is hydrophobic and rapidly adsorbs to the producer cells, or alternatively forms spontaneous aggregates. Atanassova *et al*. (2003) characterized an antimicrobial proteinaceous compound from *Lactobacillus paracasei* subsp. *paracasei* M3, with broad antibacterial activity and with fungistatic effects against 4 of the 26 tested

yeast species. The authors describe purification difficulties, but eventually isolated and sequenced a hydrophobic protein of approx. 43 kDa, having maximum activity at pH 6. Atanassova *et al*. (2003) called the protein a 'bacteriocin-like substance', as it does not fit the standard criteria defining bacteriocins (Nes *et al*., 1996). Bacteriologists, as well as mycologists, tend to stick to their 'kingdoms' and it is likely that a careful reevaluation of other 'bacteriocins' might reveal further antifungal activities.

Reuterin

Addition of glycerol to an overlay assay (Magnusson & Schnürer, 2001) gave a dramatic increase of the inhibitory effect of *L. coryniformis* strain Si3 against several filamentous fungi and yeasts (Magnusson, 2003). Most LAB lack the oxidative pathway of glycerol degradation, hence glycerol cannot be metabolized as a sole carbon source (Slininger, Bothast, & Smiley, 1983). According to the literature, the only pathway for glycerol degradation present in LAB goes through the intermediate state of 3-hydroxypropionaldehyde (reuterin, 3-HPA).

Reuterin, a broad-spectrum antimicrobial substance originally described from *Lactobacillus reuteri*, is one of the most intensively studied low-molecular-mass inhibitory compounds of LAB (Axelsson, Chung, Dobrogosz, & Lindgren, 1989; Chung, Axelsson, Lindgren, & Dobrogosz, 1989; Nakanishi *et al*., 2002; Talarico, Casas, Chung, & Dobrogosz, 1988). Reuterin is produced from glycerol by starving cells under anaerobic conditions, and the active reuterin is an equilibrium mixture of monomeric, hydrated monomeric and cyclic dimeric forms of 3-HPA (Talarico *et al*., 1988). Reuterin is active against several different types of microorganisms, including Gram-positive and Gram-negative bacteria, yeast and fungi. Antifungal activity was shown against species of *Candida*, *Torulopsis*, *Saccharomyces*, *Aspergillus* and *Fusarium* (Chung *et al*., 1989). The production of reuterin (3-HPA) has also been reported from *L. brevis* and *L. buchneri* (Schütz & Radler, 1984), *L. collinoides* (Claisse & Lonvaud-Funel, 2000) and *L. coryniformis* (Magnusson, 2003; Nakanishi *et al*., 2002).

A sourdough isolate of *L. reuteri* has also been shown to produce the antibiotic reutericyclin, a tetramic acid active against many Gram-positive bacteria, including common sourdough LAB, but lacking activity against yeast (Gänzle, Höltzel, Walter, Jung, & Hammes, 2000; Höltzel, Gänzle, Nicholson, Hammes, & Jung, 2000).

Glycerol addition to all *L. coryniformis* strains investigated dramatically increased their antifungal activity (Magnusson, 2003). During isolation of glycerol metabolites from *L. coryniformis* we detected equal amounts of 3-hydroxypropionic acid and 1,3-propanediol, and only trace amounts of 3-HPA, similar to what Sobolov and Smiley (1960) found with *Lactobacillus* sp. strain 208-A. They proposed a mechanism for glycerol breakdown by LAB through dehydration of glycerol to 3-HPA (Sobolov & Smiley, 1960), that might be oxidized further to 3-hydroxypropionic acid or reduced to 1,3-propanediol. The first step is catalysed by a glycerol dehydratase and the second step by a NAD-linked reductase, whereas the oxidation to 3-hydroxypropionic acid appears to be spontaneous (Sauvageot, Muller, Hartke, Auffray, & Laplace, 2002; Sobolov & Smiley, 1960). The glycerol dehydratase of *L. reuteri* has been purified and characterized (Talarico & Dobrogosz, 1990) and recent genetic and biochemical data suggests that LAB glycerol/diol dehydratases are structurally similar to the corresponding enzymes of other bacterial genera (Sauvageot, Muller *et al*., 2002; Sauvageot, Pichereau *et al*., 2002).

Purification of antifungal low-molecular-mass compounds

We devised a procedure for purification of hydrophobic low-molecular-mass inhibitory compounds from cell free supernatants of LAB cultures, including solid phase extraction (SPE) and C_{18}-column high pressure liquid chromatography (HPLC) (Magnusson *et al*., 2003; Sjögren *et al*., 2003). Sterile filtered supernatant of LAB was separated on a solid phase C_{18} extraction column. The hydrophilic water phase containing most of the lactic and acetic acid was not evaluated further. The hydrophobic fraction was separated on a C_{18} reversed phase column, with acetonitrile as mobile phase, and fractions were collected in a 96-well microtiter plate. The antifungal activities of the different fractions were evaluated against the indicator fungus *Aspergillus fumigatus* (Magnusson *et al*., 2003; Sjögren *et al*., 2003). Fractions with inhibitory activity were further purified in a second preparative HPLC run with a porous graphite carbon column. Structures of antifungal compounds isolated during the second HPLC run were determined using nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS, Magnusson *et al*., 2003; Sjögren *et al*., 2003; Ström, Sjögren, Broberg, & Schnürer, 2002). The bioassay-guided isolation of antifungal compounds was highly reproducible, and different LAB species gave very specific inhibition patterns (Magnusson, 2003; Magnusson *et al*., 2003; Sjögren *et al*., 2003).

Fatty acids

Using the bioassay-guided isolation protocol described above, we discovered several 3-hydroxylated fatty acids with antifungal activity from *Lactobacillus plantarum* MiLAB 14 (Sjögren *et al*., 2003). No previous reports on the antifungal activity of hydroxylated fatty acids produced by LAB are available. The 3-hydroxylated fatty acids from MiLAB 14 were all isolated from the supernatant.

Lipolytic LAB can produce significant amounts of antimicrobial fatty acids that also contribute to the sensory quality of fermented foods (Earnshaw, 1992). Rao and Reddy (1984) found several fatty acids from cultures of LAB in fermented milk. While investigating straight-chained fatty acids, Woolford (1975) observed that

antimicrobial activity increases with chain length. Caprylic (C_8) acid and longer fatty acids are generally the most effective (except undecanoic, C_{11}). Woolford (1975) noted that acids longer than 10 carbons were difficult to solve in water solutions. Baird-Parker (1980) also concluded that the antimicrobial activity of organic acids generally increased with chain length, but due to low solubility in water, aliphatic acids longer than C_{10} or C_{11} were not as effective as antimicrobial compounds. Kabra (1983) on the other hand found that fatty acids with 12–16 carbons were most effective and exhibited detergent-like properties. We observed that a hydroxylated fatty acid with 12 carbons had the strongest antifungal activity (Sjögren *et al*., 2003).

Bergsson, Armfinnsson, Steingrimsson, and Thormar (2001) investigated the effect of fatty acids and monoglycerides on the growth of *C. albicans*. They found that when yeast cells were treated with 10 mM of the fatty acids, it was only capric (C_{10}) and lauric (C_{12}) acid that inhibited the yeast, which agrees with the data of Woolford (1975). Corsetti *et al*. (1998) discovered that a *Lactobacillus sanfranciscensis* isolate from sourdough produced a mixture of organic acids with antimould activity. Caproic (C_6) acid played a key role, but propionic, butyric and valeric acids also contributed to the inhibitory effect.

We found that the hydroxylated fatty acids had strong antifungal activity against a broad spectrum of yeasts and moulds (Sjögren *et al*., 2003). Yeasts were generally more sensitive to the fatty acids than moulds. The minimum inhibitory concentrations (MIC) of the hydroxylated fatty acids against moulds and yeasts ranged between 10 and 100 μg ml^{-1} (Sjögren *et al*., 2003). This could be compared with standard antifungal drugs, *e.g.* amphotericin B that inhibits fungal growth at concentrations in the μg ml^{-1} range (McGinnis & Rinaldi, 1991).

Production of hydroxylated fatty acids followed bacterial growth, indicating that they do not result from cell lysis (Sjögren *et al*., 2003). The metabolic role of these hydroxylated fatty acids is not clear, and their potential antifungal activities in natural ecosystems are not known.

Phenyllactic acid

Lavermicocca *et al*. (2000) reported the production of phenyllactic acid and 4-hydroxy-phenyllactic acid from *L. plantarum* 21B, a sourdough isolate with antifungal activity against several species of filamentous fungi. Phenyllactic acid has also been identified from culture supernatants of *L. plantarum* MiLAB 393 (Ström *et al*., 2002), *L. coryniformis* strain Si3, and strains of *Pediococcus pentosaceus* and *L. sakei* (Magnusson *et al*., 2003). Phenyllactic acid is only active against yeasts and moulds at mg ml^{-1} concentrations. However, this metabolite will most certainly contribute to the overall antifungal effect in synergy with other compounds produced by LAB. Indeed, in sourdough bread started with *L. plantarum* 21B the onset of growth of the mould *Aspergillus niger* was delayed 7 days, compared to bread started with a *Lactobacillus brevis* that did not produce phenyllactic acid (Lavermicocca *et al*., 2000).

Cyclic dipeptides and other low-molecular-mass inhibitory compounds

Niku-Paavola *et al*. (1999) discovered new types of antimicrobial compounds from the culture filtrate of *L. plantarum* VTT E-78076. The active fraction included benzoic acid, 5-methyl-2,4-imidazolidinedione (methylhydantoine), tetrahydro-4-hydroxy-4-methyl-2H-pyran-2-one (mevalonolactone), and cyclo(glycyl-L-leucyl). Ström *et al*. (2002) found two cyclic dipeptides cyclo(Phe-Pro) and cyclo(Phe-OH-Pro) in the supernatant of *L. plantarum* MiLAB 393. The antimicrobial effect of several different cyclic dipeptides has been investigated (Gänzle *et al*., 2000; Graz, Hunt, Jamie, & Milne, 1999; Graz, Jamie, Versluis, & Milne, 2001). Magnusson *et al*. (2003) found that cyclo(Phe-Pro) and cyclo(Phe-OH-Pro) are also produced by strains of *P. pentosaceus*, *L. sakei* and *L. coryniformis* and thus might be common LAB metabolites. The cyclic dipeptides have antifungal activity at mg ml^{-1} concentrations, and hence are much less effective than the hydroxylated fatty acids described above. We have recently found low concentrations of several cyclic dipeptides present in MRS (broth and agar) and other complex LAB media. However, the amounts detected are not high enough to exert antimicrobial activity in the bioassays used during purification (unpublished results).

Conclusion and future trends

The research field of antifungal LAB is still very novel. Until recently, most publications on the antifungal activity of LAB have merely illustrated their inhibitory effects, but have seldomly identified active compounds or other reasons for the inhibitory activity. To date, the majority of antifungal substances purified from LAB have been low-molecular-mass compounds, *e.g.* phenyllactic acids, cyclic dipeptides and short or medium chain fatty acids. Only three publications have presented clear evidence for the production of antifungal peptides/proteins from LAB.

Many antifungal LAB produce several active compounds (Fig. 1, Table 1) and it is likely that synergistic effects exist between them. Among these the hydroxylated fatty acids appear to be produced in low amounts.

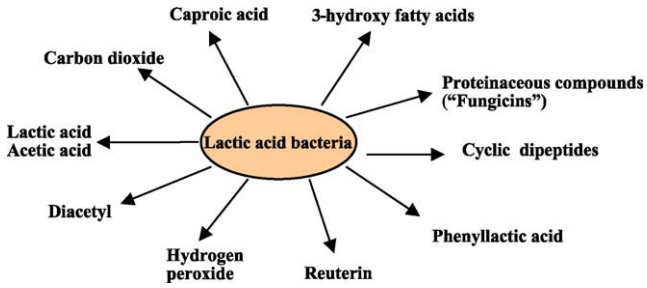

Fig. 1. Summary of current knowledge of the main antifungal compounds produced by different LAB.

They could be detected only after development of new procedures for the isolation of antifungal metabolites. Many other low-molecular-mass compounds might remain undetected due to the lack of efficient technology for isolating very small amounts of active substances. More time and energy should thus be devoted to the development of sensitive methods for detecting antifungal compounds in complex environments.

Studying specific interactions between LAB and fungi is complicated by the general antimicrobial effects of the bacterial fermentation products (lactic acid, acetic acid), that might act synergistically with more targeted antifungal compounds. In nature, this is likely to be important for the outcome of microbial interactions. During preservation of food and in other biotechnological applications, each antimicrobial compound produced during LAB fermentation will provide an additional hurdle for spoilage microorganisms.

Evidence now suggests that antifungal LAB or their metabolites can substitute traditional chemical preservatives in complex environments. The use of a phenyllactic acid producing *L. plantarum* strain as sourdough starter culture was found to prolong shelf life and prevent mould growth on bread baked from the sourdough (Lavermicocca *et al*., 2000). However, to enable use of antifungal LAB to control mould growth in bread baked from sourdough, the bacteria must not reduce the leavening power of *Saccharomyces cerevisiae*. Although no direct studies on this have been done, several authors have found that compounds active against filamentous fungi, *i.e.* moulds, do not necessarily inhibit yeasts.

Another promising example of LAB as biopreservatives is that inoculation with an antifungal *L. plantarum* strain could improve aerobic stability and reduce yeast and mould counts in grass silage, thus replacing the chemical preservative sodium benzoate (K. Holmgren, Medipharm AB, pers. comm.). If successfully applied during silage storage this might reduce the risk of mycotoxin poisoning of animals and prevent further contamination of food products.

For many years consumers have requested food preserved without chemical additives. The use of antifungal LAB instead of chemical preservatives would enable the food industry to grant this request. However, the introduction of large-scale biopreservation of food requires careful safety assessment and risk analysis as part of a formal notification or registration procedures. Given the very limited resources spent to date on investigations of antifungal LAB, it is likely that many new antifungal compounds await discovery. It is also reasonable to expect that combinations of LAB species, or strains producing different antifungal compounds, could be employed as novel biopreservatives during storage of both food and animal feeds.

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