Abstract

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Enrichment culture techniques produced more than 1200 isolates of lactic acid bacteria (LAB) that were screened for antifungal activity against the indicator mould *Aspergillus fumigatus*. Approximately 10% of the LAB were active, but only 4% had medium or strong activity in an agar plate assay. The majority of isolates with strong antifungal activity were *Lactobacillus coryniformis* strains, but *Lactobacillus plantarum* and *Pediococcus pentosaceus* were also frequently identified. Some of the isolates lost activity during storage but most maintained their fungal inhibitory effect. Large variations in sensitivity were observed between different moulds and yeasts. Antifungal cyclic dipeptides and phenyllactic acid were detected in culture filtrates from several of the LAB isolates.

Lactobacillus coryniformis subsp. *coryniformis* strain Si3 produced an antifungal compound that lost activity when treated with proteases. The antifungal peptide(s) was heat stable, with a size of approx. 3kDa and had maximum activity at pH 3.0 to 4.5. Addition of ethanol to the growth medium of strain Si3 prevented a decline in observed antifungal activity during the stationary phase. Glycerol addition to agar plates with *L. coryniformis* strains, overlaid with soft agar suspensions of yeast cells or fungal spores, strongly enhanced the antifungal effect. This was particularly true with spoilage moulds and yeasts, *e.g. Penicillium roqueforti* and *Pichia anomala*, not normally affected by the antifungal metabolites of *L. coryniformis*. Chemical and genetic data suggested that reuterin (3-hydroxypropionaldehyde) was the cause of this effect. The glycerol/diol dehydratase operon of *L. coryniformis* was partially elucidated and found to be similar to that *Lactobacillus collinoides*.

Bioassay-guided isolation of new metabolites from LAB revealed that *Lactobacillus plantarum* MiLAB 14 produces hydroxylated fatty acids with strong antifungal effects. 3-Hydroxydecanoic acid, 3-hydroxydodecanoic acid, 3-hydroxytetradecanoic acid and 3-hydroxy-5-*cis*-dodecenoic acid were characterized from the supernatant of MiLAB 14. The hydroxy fatty acids had total inhibitory effects in the range 10 to >100 μg ml^{-1} against several moulds and yeasts.

Keywords: antifungal, peptides, cyclic dipeptides, phenyllactic acid, hydroxy fatty acids, reuterin, 3-HPA, 3-hydroxypropionic acid, yeast, mould, fungi

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“A man who dares to waste an hour of life has not discovered the value of life"

- Darwin

Contents

Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I Magnusson, J., K. Ström, S. Roos, J. Sjögren, and J. Schnürer. (2003). Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. FEMS Microbiology Letters 219:129-135.

II Magnusson, J. and J. Schnürer. (2001). *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 produces a broad-spectrum proteinaceous antifungal compound. Applied and Environmental Microbiology. 67:1-5.

III Sjögren, J., J. Magnusson, A. Broberg, J. Schnürer and L. Kenne. (2003). Antifungal 3-hydroxy fatty acids from *Lactobacillus plantarum* MiLAB 14. (Manuscript)

IV Magnusson, J., S. Roos, A. Broberg, H. Jonsson and J. Schnürer. (2003). Glycerol increases the antifungal activity of *Lactobacillus coryniformis*: genetic and chemical support for involvement of 3-hydroxypropionaldehyde (reuterin). (Manuscript)

Aims

This thesis is an attempt to elucidate the complex nature of the antifungal activities of lactic acid bacteria. The primary hypothesis was that it should be possible to isolate lactic acid bacteria from natural habitats that inhibit growth of moulds and yeasts, through the production of substances other than lactic acid. Early during the investigations, it became clear that the focus of the work and the thesis itself would be the specific active compounds that were produced and not the effect on the fungus. This was mainly due to the surprisingly high number of antifungal compounds and the complexity of the antifungal effect.

The results are based on investigations of selected strains from more than 1200 isolates of lactic acid bacteria from different environments. The overall hypothesis of this work can be divided into the following objectives:

- Establish methods for determination of antifungal activity (**I, II**)
- Isolate and identify lactic acid bacteria from natural environments that exert anti mould and/or anti yeast effects (**I**)
- Characterize the antifungal spectra of lactic acid bacteria (**I, II**)
- Isolate and chemically characterize antifungal compounds from lactic acid bacteria and determine the individual and synergistic effect against moulds and yeast. (**II, III, IV**)

Introduction

Lactic acid bacteria (LAB) were used in food and feed fermentation processes long before any knowledge of the presence of the bacteria themselves existed. It is impossible to say when man first used these processes, but old Egyptian murals show the production of silage several thousand years ago and fermentation as a preservative measure for food has also been present since historical times. The use of fermentation processes has increased during the centuries and includes many different kinds of animal feed and food (Ross, Morgan & Hill, 2002). However, it was only during the last century that we began to understand the metabolic processes behind the preserving effect of lactic acid bacteria. During the last century it was realized that the lactic acid bacteria are responsible for the fermentation, and through that the (bio)preservative effect, utilized in many food and feed processes. Biopreservation refers to extended shelf life and enhanced safety of foods using the natural or added micro-flora and their antimicrobial products.

Lactic acid bacteria 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, by tradition, been established as a natural, consumer and environment friendly way of preserving food and feed. The preserving effect is mainly due to the reduction of pH through the production of

lactic acid. Besides lactic acid, several other antimicrobial compounds are produced during growth of LAB (Lindgren & Dobrogosz, 1990). Research dealing with fungal inhibition by lactic acid bacteria and the compounds produced by these bacteria is still novel. While the number of publications regarding antibacterial activity of LAB is large, our knowledge of the antifungal activities of these bacteria is still limited.

The combination of the increase of antibiotic resistance and a more general chemical resistance of pathogenic and/or spoilage organisms, together with an increasing general awareness among consumers calls for alternative measures to preserve food (Brul & Coote, 1999). There is a definite need for new ways to extend shelf life, suppress fungi, and produce safe, preservative free food. This need stimulates the search for lactic acid bacteria with these abilities. The long tradition of using lactic acid bacteria when processing food and feed, in combination with the more recent knowledge on positive health effects caused by ingestion of probiotic LAB (Mattila-Sandoholm, Mättö & Saarela, 1999; Andersson *et al.,* 2001), makes them an promising alternative.

Fungal spoilage of food and feed

Ergotism, or St. Anthony´s fire (originally "Holy Fire"), was documented as a common human disease around the 10:th century. The origin of the disease remained a mystery until the mid 19:th century when it was discovered that it was caused by production of the ergot toxin from the fungus *Claviceps purpurea* on cereals. St. Anthony's fire is a toxic condition with symptoms including hallucinations, disorientation, muscle cramps, convulsions, miscarriages, and gangrene and may result in death. The large consumption of grain infected with sclerotia of *C. purpurea* caused several thousand deaths in the 10:th and 11:th century in central Europe and these fungal infections were possibly the reason for reduced fertility in Europe prior to 1750 (Pohland, 1993; van Dongen & de Groot, 1995; Packer, 1998). In the mid 20:th century another fungal toxin, aflatoxin, was reported from the fungus *Aspergillus flavus* (Filtenborg, Frisvad & Thrane, 1996). Several of the aflatoxin derivates have strong carcinogenic properties. They can be formed on a number of substrates, such as oilseeds and nuts, and certain subtropical fruits, *e.g.* figs. In the mid 1960´s, an outbreak of aflatoxin poisoning occurred in Great Britain, commonly called "Turkey X-disease", that lead to the death of more than 100 000 turkeys (van Egmond, 2000). Today, more than 400 mycotoxins are known from many different fungal genera and the number is increasing rapidly (Filtenborg, Frisvad & Thrane, 1996).

In addition to health hazards from mycotoxins, contaminating moulds cause considerable general spoilage of food and feed. Filamentous moulds and yeasts are common spoilage organisms of food products as fermented milk products, cheese, bread, stored crops and feed such as hay and silage (Bullerman, 1977; Moon, 1983; Bonestroo *et al*., 1993; Filtenborg, Frisvad & Thrane, 1996). It is estimated that between 5 and 10% of the world's food production is lost due to fungal

detoriation (Pitt & Hocking, 1999). In Western Europe, mould spoilage of bread alone is estimated to cause an annual economical loss of about £242 million (Corsetti *et al*., 1998).

Penicillium and *Aspergillus* species have been reported as spoilage organisms during storage 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, Frisvad & Thrane, 1996; Samson *et al*., 2000). Different *Penicillium* species frequently occur in foods stored under cold conditions, and *P. roqueforti* and *P. commune* commonly spoil hard cheese (Filtenborg, Frisvad & Thrane, 1996, Pitt & Hocking, 1999). Yeasts such as *Candida parapsilosis*, *Rhodotorula mucilaginosa*, *Kluyveromyces marxianus* and *Debaromyces hansenii* are common spoilage organisms of youghurt and other fermented dairy products (Pitt & Hocking, 1999; Loureiro & Querol, 1999).

However, yeasts also play an essential role in the preparation of certain dairy products and in the ripening of various cheeses where they contribute substantially to the characteristics of the final product. Yeasts contribute to the fermentation by supporting the lactic acid bacterial starter culture, inhibiting unwanted microorganisms, or adding desirable biochemical changes to the final product *e.g.* cheese (Viljoen, 2001). Some moulds *e.g. P. roqueforti* and *P. camemberti* are also essential for the production of soft cheese (Samson, 2000).

Traditional fungal control and the search for a natural alternative

Several techniques are used for the preservation of food and feed. 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). Many organic acids are used as food additives, the most active are acetic, lactic, propionic, sorbic and benzoic acids (Brul & Coote, 1999). Both sorbic and benzoic acid have a broad spectrum of activity (Nielsen & Deboer, 2000; Davidson, 2001). Benzoic acids and sodium benzoate are used primarily as antifungal agents (Davidson, 2001). Natamycin is an antibiotic agent produced by *Streptomyces natalensis* that is very effective against yeasts and moulds and often used as preservative on surfaces of hard cheese (Davidson, 2001).

It is a well-known fact that increasing amounts of microorganisms are becoming resistant to antibiotics. Fungi are no exception and more species of both human fungal pathogens and spoilage moulds in food and feed systems are becoming resistant. However, 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; Viljoen, 2001; Sanglard, 2002). High initial mould populations can degrade

sorbic acid in cheese and a number of *Penicillium*, *Saccharomyces* and *Zygosaccharomyces* species can grow in presence of and degrade potassium sorbate (Davidsson, 2001). The number of moulds degrading sorbate is increasing (Nielsen & Deboer, 2000). In addition, isolates of *P. roqueforti* have been found to be resistant to benzoate (Nielsen & Deboer, 2000).

The mould *Penicillium discolor* has recently acquired resistance to chemicals used in food processing, *e.g.* natamycin (Nielsen & Deboer, 2000; Cabo, Braber & Koenraad, 2002). *P. discolor* can grow on high concentrations of natamycin and cause spoilage on hard cheese (Filtenborg, Frisvad & Thrane, 1996, Nielsen & Deboer, 2000). Yeast like *Debaromyces 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. It is therefore essential to develop good alternatives.

The general public wants to reduce the use of chemical preservatives or additives in food or feed. Instead, consumers require high quality, preservative free, safe but mildly processed food with extended shelf life (Brul & Coote, 1999). This is of course not an easy task to solve. In addition, present legislation has restricted the use of some currently accepted preservatives in different foods (Brul and Coote 1999). This is also one important aspect to bear in mind when discussing the need for novel preservation methods to inhibit the growth of unwanted fungal contamination.

Lactic acid bacteria occur naturally in foods or are added as pure cultures to various food products. They are considered to be harmless or even to have an advantage for human health (probiotics). LAB have a GRAS affirmation (generally recognized as safe) and have, through tradition, been established as a natural consumer and environmental friendly way to preserve food and feed. 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 for their use as starter cultures in the manufacture of dairy products such as acidophilus milk, yoghurt, buttermilk, cottage cheeses, hard cheeses (Cheddar and Edam) and soft cheeses (Brie and Camembert) among others (Carr, Chill & Maida, 2002). Lactic acid bacteria used in milk fermentations, especially *Lactococcus lactis* are among the best characterized in the group. *Lactobacillus* and *Leuconostoc* species are also associated with milk products (Stiles, 1996).

The coexistence of lactic acid bacteria and fungi is essential for the success of several biotechnological applications *e.g.* sourdough bread making, where the ratio of lactic acid bacteria/yeast is generally 100:1 (Gobbetti, 1998). Even though LAB are essential for the production of several fermented food and feed products they can also exist as spoilage organisms causing economical loss. Makanjuola, Tymon & Springham (1992) found that *Lactobacillus plantarum* caused flocculation of fermentation yeast, and thereby reduced ethanol production in whisky fermentations. *Lactobacillus* and *Pediococcus* species have been reported to act as

spoilage organisms during fermentation of beer (Jespersen & Jakobsen, 1996), and certain species of *Lactobacillus* are known to spoil wine and cider through the production of acrolein (Claisse & Lonvaud-Funel 2000).

What is a lactic acid bacterium?

Taxonomy

The term lactic acid bacteria (LAB) was gradually accepted in the beginning of the 20:th century. Other terms as "milk souring" and "lactic acid producing" bacteria had previously been used for the same bacteria causing a slight confusion. This ended with publication of a monograph about lactic acid bacteria written by Orla-Jensen, 1919, a work that had great impact on the systematics of LAB. Classification of LAB genera was based on morphology, mode of glucose fermentation, growth at certain temperatures, and range of sugar utilization. Even though the taxonomy has been revised since then, characters used by Orla-Jensen, are still very important in current classification of LAB (Axelsson 1998).

Lactic acid bacteria constitute a group of bacteria that have morphological, metabolic and physiological similarities, and they are also relatively closely related phylogenetically. The general description of the bacteria within the group is Gram-positive, non-sporulating, non-respiring cocci or rods, which do, through fermentation of carbohydrates, produce lactic acid as their major end product. The common agreement is that there is a core group consisting of four genera; *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus*. Recent taxonomic revisions have proposed several new genera and the remaining group now comprises the following: *Aerococcus, Alloiococcus Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactococcus, Oenococcus, Tetragenococcus, Vagococcus*, and *Weissella*. Lactobacilli, carnobacteria and some weissella are rods while the remaining genera are cocci (Axelsson, 1998).

For identification of lactic acid bacteria, phenotypic methods have been most commonly used. More recently, genetic techniques, such as 16S rDNA sequencing have been developed which allows a more consistent and accurate identification of individual strains. Determination of short sequences of 16S rDNA is today used as a simple way for species determination of isolates of lactic acid bacteria (Schleifer & Ludwig, (1995).

Metabolism

Lactic acid bacteria have two different metabolic pathways for hexose fermentation, the homo- and heterofermentative pathway. The homofermentative pathway follows the glycolysis (Embden-Meyerhof-Parnas pathway), and occurs among *Lactococcus*, *Streptococcus*, *Pediococcus*, and homofermentative *Lactobacillus* species. It is characterized by the splitting of fructose-1,6-diphosphate (FDP) into two triose phosphate moieties, glyceraldehyde-3-

phosphate (GAP), and dihydroxyacetonephosphate (DHAP) in equilibrium, by a FDP aldolase. GAP is further converted to pyruvate, which is then reduced to lactic acid (Axelsson, 1998; Kandler, 1983).

The heterofermentative, or the 6-phosphogluconate / phosphoketolase pathway is characterized by the oxidation of glucose 6-phosphate to gluconate 6-phosphate followed by decarboxylation. The remaining pentose is split into a C-3 moiety (glyceraldehyde-3-phosphate) and a C-2 moiety (acetyl-phosphate) by a phosphoketolase. As a result, equimolar amounts of CO_2, lactate and ethanol are formed from hexose. Heterofermentative lactic acid bacteria generally ferment pentoses, although there are some pentose negative strains (Kandler, 1983). Heterofermentative LAB also possesses the ability to use external electron-acceptors to regenerate NADH, and hence gain more energy. Because of this alternative pathway, acetate will be formed instead of ethanol (Axelsson, 1998). One group of lactic acid bacteria has the ability to use both pathways but prefers the homofermentative pathway in presence of hexoses (Axelsson, 1998; Kandler, 1983).

Homofermentation

1 Hexose + 2ADP + 2Pi → 2 Lactate + 2 ATP

Heterofermentation

1 Hexose + 1 ADP + Pi → Lactate + Ethanol + CO_2 + 1 ATP

or (if external e- acceptor)

1 Hexose + 2 ADP + Pi → Lactate + Acetate + CO_2 + 2 ATP

Habitats

Lactic acid bacteria are commonly found and known to proliferate in fermentation processes or as colonizers of mucosal surfaces of higher animals. They require a nutrient rich environment in order to establish. Besides carbohydrates, they also need to be supplied with amino acids, peptides, salts and vitamins among others (Hammes, Weiss & Holzapfel, 1992; Carr, Chill & Maida, 2002).

Antifungal activities of lactic acid bacteria

Lactic acid bacteria produce a variety of compounds with antimicrobial activity. Lactic and acetic acids are produced as end products during lactic acid bacterial fermentation causing a reduction in pH, but other substances such as hydrogen peroxide, formic acid, propionic acid, acetoin and diacetyl, are also produced (Lindgren & Dobroqosz, 1990).

The precise mechanism of antimicrobial action can often not be defined because of a complex interaction between different compounds. Synergistic effects are often seen between the compounds involved in the antimicrobial action (Corsetti *et al*., 1998; Niku-Paavola *et al*., 1999). Much research has 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 fact that some fungi are sensitive to the normal by-products of LAB-metabolism, most notably lactic and acetic acids (Lindgren & Dobroqosz, 1990; Piard & Desmazeaud, 1992; Bonestroo *et al*., 1993).

In the following sections, the inhibitory compounds are described under three headings; fermentation products, proteinaceous compounds and low molecular weight inhibitory compounds. The section ends with a summary of the current knowledge of antifungal compounds from LAB. The literature review is combined with a summary of results obtained within this thesis work. The number of publication about antifungal activity of lactic acid bacteria is low. Reviewing the area reveals the fact that a majority of the publications describe activity of lactic acid bacteria, and that only very few present any characterization of compounds or mechanisms. A summary of publications on antifungal lactic acid bacteria is presented in table 1.

Table 1. Compilation of publications reporting antifungal activity of lactic acid bacteria (ND = Not determined)

LAB isolate*	Activity spectrum	Compound(s)	Reference
Streptococcus lactis C10	*Aspergillus parasiticus*	ND	Wiseman & Marth (1981)
Lactobacillus casei ATCC 393	*Aspergillus parasiticus*	ND	El-Gendy & Marth (1981)
S. lactis	*Aspergillus flavus*	ND	Coallier-Ascah & Idziak (1985)
L. casei var. *rhamnosus*	Broad spectrum	ND	King & Vanderbergh (1986)
L. casei var. *rhamnosus*	Broad spectrum	< 1kDa	Vanderbergh & King (1988)
Lactobacillus reuteri	Broad spectrum	3-HPA (reuterin)	Talarico *et al.* (1988); Chung *et al.* (1989)
Lactobacillus plantarum	Unspecified spoilage mould	ND	Hill (1989)
S. lactis subsp. *diacetilactis* DRC1	*Aspergillus fumigatus, Aspergillus parasiticus, Rhizopus stolonifer,*	Possibly proteinaceous	Batish, Grover & Lal (1989)
Pediococcus acidilactici	*Saccharomyces cerevesiae*	Possibly proteinaceous	Vanderbergh & Kanka (1989)
Lactobacillus acidophilus R	*Aspergillus fumigatus*	ND	Batish, Lal & Grover (1990)
Lactococcus lactis	*Aspergillus parasiticus*	ND	Luchese & Harrigan (1990)
L.casei subsp. *rhamnosus* *L. plantarum* *Leuconocstoc mesenteroides*	*Penicillium* spp., *Aspergillus* spp.	ND	Suzuki, Nomura & Morichi (1991)
L. plantarum	*Saccharomyces cerevisiae*	ND	Makanjoula, Thymon & Springham (1992)
L.casei subsp. *rhamnosus* LC-705	*Candida lusitaniae*, *Aspergillus* niger, *Fusarium* spp., *Penicillium* spp., *Cladosporium* spp.	ND	Mäyrä-Mäkinen *et al.* (1994)
L. lactis subsp. *lactis* CHD 28.3	*Aspergillus flavus*, *A. parasiticus*, *Fusarium* spp.	Possibly proteinacious	Roy et al. (1996)
L. casei	*Penicillium* spp.	Possibly proteinaceous	Gourama (1997)

LAB isolate*	Activity spectrum	Compound(s)	Reference
Lactobacillus casei subsp. *pseudoplantarum*	*Aspergillus flavus*	Possibly proteinaceous, <1kDA	Gourama & Bullerman (1995; 1997)
Lactobacillus sanfrancisco 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 (2001)
L. rahmnosus	*Penicillium* spp., *Aspergillus* spp., *Fusarium* spp., *Alternaria* spp.	Sodium acetate[1]	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	**I, II, IV**
L. plantarum MiLAB14	Broad spectrum	Hydroxy fatty acids, phenyllactic acid, cyclo(Phe-Pro), cyclo(Phe-OH-Pro),	**II, III**, Unpublished results
Pediococcus pentosaceus MiLAB 24	Broad spectrum	Cyclo(phe-OH-Pro)	Unpublished results,

* Some species have through taxonomic revisions, received new species identities, which are not taken into account here.

[1.] 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.

Fermentation products

Organic acids

The major metabolite of lactic acid bacteria is lactic acid, which is responsible for the significant pH changes in their growth environment – sufficient to antagonize many microorganisms (Eklund, 1989). The undissociated form of the weak organic acid diffuses over the cell membrane and is, depending on intracellular pH, more or less dissociated inside the cell, releasing H^+-ions that acidify the cytoplasm (Axelsson, 1990; Piard & Desmazeaud, 1991). In addition to the pH effect, the undissociated form of the molecule mediates the antimicrobial effect of collapsing the electrochemical proton gradient causing bacteriostasis and eventual death of the susceptible bacteria (Eklund, 1989). The effect is more pronounced at pH values below the pK_a value of the acid, *i.e.* when the acid is in undissociated state (Axelsson, 1990; Piard & Desmazeaud, 1991). Reiss (1976) observed that 0.75% lactic acid (approx. 80 mM) reduced the growth of *Aspergillus parasiticus*, while El-Gazzar, Rusul & Marth, (1987) found that lactic acid up to concentrations of 2% (200 mM) supported the growth of *Aspergillus parasiticus*. This difference could be due either to different tolerance to organics acids between strains or to variations in their experimental designs.

Heterofermentative LAB produces acetic acid in 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 acid interact with cell membranes to neutralise the electrochemical proton gradient, but the effect of acetic and propionic acid is often dependent on the decrease in pH caused by lactic acid (Freese, Sheu & Galliers, 1973; Eklund, 1989). Propionic acid negatively influence 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 (Freese, Sheu & Galliers, 1973; Eklund, 1989). Some 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 high concentrations of lactic, acetic and propionic acid inhibited yeast species that normally grow well in relatively high concentrations (100 mM) of the individual acids, except for propionic acid.

The combination of lactic acid produced during LAB growth and the sodium acetate of de Man, Rogosa, Sharpe (MRS) substrate (De Man, Rogosa & Sharpe, 1960) a standard growth medium for LAB, has synergistic antifungal effects. (Cabo, Braber & Koenraad, 2002; Stiles *et al*., 2002). The sodium acetate in MRS might also have synergistic effects with additional antifungal compounds produced by LAB (Stiles *et al*., 2002). Similar effects have been detected in this work (unpublished). However, we also found that non-inhibiting lactic acid bacteria produced more lactic acid than highly active ones (**I**). Anyhow, the inhibitory effects of organic acids such as lactic, acetic and propionic acid will continue to

complicate studies on antimicrobial effects of lactic acid bacteria, unless rigorous further purification and characterization of substances is applied (**I, II, III, IV**).

Other end products

Most LAB possess flavoprotein oxidases (and NADH peroxidases), which enables them to produce hydrogen peroxide (H_2O_2) in the presence of oxygen. Hydrogen peroxide accumulates in the environment since LAB not produces catalase (Condon, 1987). The antimicrobial effect of hydrogen peroxide is well documented (Davidsson *et al*., 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, even though the concentration itself is not inhibitory, may be potentiated by the presence of lactoperoxidase and thiocyanate in natural environments such as milk and saliva (Condon, 1987). The lactoperoxidase-thiocyanate-peroxide system involves the reaction of hydrogen peroxide and thiocyanate through the catalysis by lactoperoxidase. The intermediary products such as hypothiocyanate act inhibitory to 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 *et al*., 1975; Björck, 1978). Fitzsimmons & Berry (1994) reported the inhibitory effect of this system against *Candida albicans*. Rodríguez *et al*. (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) is the molecule responsible for the characteristic aroma associated with butter. It is produced by strains of all genera of lactic acid bacteria during citrate fermentation (Earnshaw, 1992). The antimicrobial effect of diacetyl is well documented, especially at pH below 7.0 (Jay, 1982). However, the amount of diacetyl needed to exert antimicrobial activity (close to 200 mM) will dramatically alter the taste and aroma of the product (Piard & Desmazeaud, 1991).

Proteinaceous compounds

Ribosomally synthesised antimicrobial peptides are found in a broad range of organisms, such as mammals, birds, amphibians, insects, plants and microorganisms. Although the group of antimicrobial peptides is diverse, they generally share some features; a hydrophobic and a hydrophilic end, a size of 20-50 amino acids, and cationic properties (Nissen-Meyer & Nes, 1997; Hildeng-Hauge, 1998). LAB produce antibacterial, ribosomally synthesised, peptides, generally termed bacteriocins (Nes *et al*., 1996). Already in 1928, Roger and Whittier reported the first well-documented antimicrobial peptide, nisin, produced by a strain of *Lactococcus lactis* (Hirch *et al*., 1951).

A large number of bacteriocins have been characterized from lactic acid bacteria in recent years. The bacteriocins from lactic acid bacteria are commonly divided into three groups: class I – the lantibiotics; class II – the heat stable unmodified

bacteriocins; class III the larger heat stable bacteriocins (Nes *et al*., 1996; Nes & Holo, 2000). These compounds are generally only active against closely related bacterial species and there is no evidence that bacteriocins have any effect on growth of yeast or moulds.

In contrast, there are only few reports on the production on antifungal peptides produced by lactic acid bacteria. Several authors have reported that the antifungal activity of LAB is lost after treatment with proteolytic enzymes. Batish, Grover & Lal (1989) claim that the antifungal substance produced by a lactic acid bacterium included in their study was of proteinaceous nature since it was degraded by proteinases. However, they did not present any results supporting these claims and did not characterise the active compound in any detail. Roy *et al*., (1996) isolated a *Lactococcus lactis* subsp. *lactis* with antagonistic activity against several filamentous fungi. After enzymatic treatment with chymotrypsin, trypsin and pronase E, the antifungal activity disappeared, indicating a proteinaceous nature of the antifungal substance. The substance was not characterized further. 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, but the compound was not characterized further.

Gourama & Bullerman (1995, 1997) showed that a commercially available silage inoculant with a combination of *Lactobacillus* species (*L. plantarum, L. bulgaricus* and *L. acidophilus*) exerted antifungal and anti-aflatoxin activity against *A. flavus*. Guorama & Bullerman (1995) proposed in their first study that the inhibitory activity was caused by a low molecular weight inhibitory compound. When investigating the commercially available starter culture they discovered that a *Lactobacillus casei* subsp. *pseudoplantarum* was responsible for the inhibitory activity. The activity was sensitive to treatments with the proteolytic enzymes trypsin and α-chymotrypsin, and their conclusion was that the activity was due to the production of a small peptide of less than 1 kDa. The activity was not investigated or characterized further. The anti-aflatoxigentic properties of lactic acid bacteria has since then found another focus since it has been shown that several types of fungal toxins adhere to cells of lactic acid bacteria (Haskard *et al*., 2001; El-Nezami *et al*., 2002).

Only two publications report the purification of antifungal proteinaceous compounds from lactic acid bacteria. Okkers *et al*. (1999) purified and characterized a medium length peptide TV35b from *Lactobacillus pentosus* with fungistatic effect against *Candida albicans*. They discovered that the peptide caused reduction in growth of *Candida albicans* and induction of pseudo hyphae. The peptide was apparently not tested for activity against moulds.

We found that a proteinaceous compound produced by *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 had antifungal effect against several moulds and against the yeasts *Debaromyces hansenii* and *Kluyveromyces marxianus* (**II**). The peptide was small (approx 3kDa), heat stable, active in the pH range 3-6 and totally inactivated by proteinase K or trypsin (**II**). The same characteristics can be found among bacteriocins of subclass II (Nes & Holo, 2000).

The production of the antifungal peptide followed the same kinetics as that of several bacteriocins (Figure 1). The production kinetics of the bacteriocins amylovorin L471, produced by *Lactobacillus amylovorus* (Callewaert *et al*., 1999), Lactosin S from *Lactobacillus sake* (Mørtvet-Abildgard *et al*., 1995) and the fungistatic peptide TV35b from *Lactobacillus pentosus* were all very similar (Okkers *et al*., 1999; Figure 1).

When a *Lactobacillus coryniformis* strain Si3 culture was supplimented with either ethanol, formic or acetic acid the total amount of the antifungal peptide increased (**II,** Figure 1). The same response was observed when cultures of *Lactobacillus amylovorus* producing amylovorin L471 were treated with ethanol or organic acids (Callewaert *et al*., 1999). During addition of ethanol or organic acids the total amount of the antimicrobial peptide increased, instead of showing a peak in production as seen without ethanol (Figure 1). The same effect was seen for the production of Lactosin S from *Lactobacillus sake* (Mørtvet-Abildgard *et al*., 1995). This suggests that the peptide is highly hydrophobic in nature and rapidly adsorbs to the producer cells or forms spontaneous aggregates. However, Nilsen, Nes & Holo (1998) found that the presence of ethanol was inhibitory to production of bacteriocins enterocin A and enterocin B from *Enterococcus faecium*. The authors speculate that ethanol attenuate the response to the induction factor.

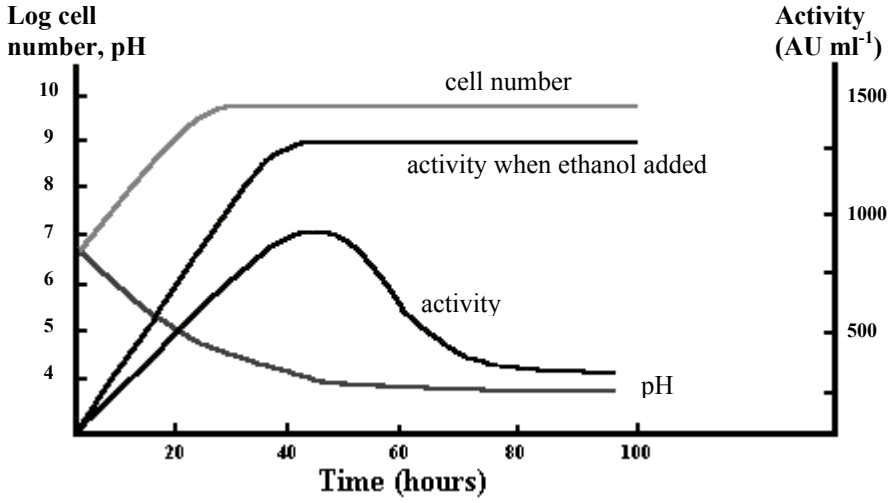


Figure 1. During normal growth, the kinetics of the production of the antifungal peptide from *L. coryniformis* strain Si3 and several bacteriocins of subclass II show similarities. The activity distinctly peaks at about 48 hours, and then rapidly decreases. When ethanol or organic acids are gradually added during growth, the activity remains high (**II;** Mørtvet-Abildgard *et al*., 1995; Callewaert *et al*., 1999).

To isolate the antifungal proteinaceous compound of strain Si3 several different purification techniques were used that gave information on the nature of the

peptide. However, we were not able to purify the protein or determine its amino acid composition. The reason might be that the substance is unstable, loosing activity after two or three purification steps or simply after storage (**II**), or that the activity is mediated by a combination of peptides, not active on their own. Similar results have been seen with bacteriocins where activity requires a complementary peptide (Anderssen *et al.*, 1998; Moll *et al.*, 1998). The observed hydrophobic nature of the antifungal peptide might also be the reason for the problems that occurred, since there is a possibility that a hydrophobic peptide might bind strongly to glass or plastic material during growth and purification. *Lactobacillus coryniformis* strain Si3 also had antibacterial activity against several closely related species at pH values below 4.5 (unpublished results).

Low molecular weight compounds

Several authors have reported the detection of antifungal low molecular weight compounds, but the number of purified and chemically characterized compounds is still low. Several antifungal low molecular weight compounds were detected in this work, and they will be described and discussed in relation to the existing literature. This chapter also includes an overview of the methods that were used in this work to isolate low molecular weight inhibitory compounds.

Reuterin

Early during this work, we discovered that accidental addition of glycerol to the overlay assay (described in paper **II**) resulted in a dramatic increase of the inhibitory effect of *Lactobacillus coryniformis* strain Si3 against several filamentous fungi and yeast. We also observed that fungi, not inhibited or inhibited to a limited extent by the "normal" antifungal effect of strain Si3, suddenly were inhibited when glycerol was present in the overlay assay (**IV**). LAB lack the oxidative pathway of glycerol degradation, hence glycerol can not be metabolised as a sole carbon source (Slininger, Bothast & Smiley, 1983). According to the literature, the only pathway for glycerol degradation present in lactic acid bacteria 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 weight inhibitory compounds (Talarico *et al*., 1988; Axelsson *et al*., 1989; Chung *et al*., 1989; Nakanishi, 2002). Reuterin is produced from glycerol by starving cells under anaerobic conditions, and the active compound reuterin is in fact 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* however, little data supporting this claim was provided (Chung *et al*., 1989).

The antifungal effect of the addition of glycerol to *L. coryniformis* strains has been investigated in this work. The results clearly showed a dramatic increase in activity when glycerol was present in the substrate (**IV**). 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 (**IV**), which is similar to what Sobolov & Smiley (1960) found for *Lactobacillus* sp. strain 208-A. They proposed a mechanism for breakdown of glycerol by lactobacilli by dehydration of glycerol to 3-HPA (Sobolov & Smiley, 1960), that further might be oxidized to 3-hydroxypropionic acid or reduced to 1,3-propandiol (Figure 2). The first step is catalysed by glycerol dehydratase and the second step by a NAD-linked reductase, whereas the oxidation to 3-hydroxypropionic acid appears to be spontaneous (Sobolov & Smiley, 1960; Savaugeot, 2002a). The glycerol dehydratase of *L. reuteri* has been purified and characterized (Talarico & Dobrogosz, 1990), but they did not present any genetic data supporting the results. However, recent genetic and biochemical results of Sauvageot *et al.* (2002a, b) suggest that the structure of lactobacilli glycerol/diol dehydratases is similar to the corresponding enzymes found in other bacterial genera.

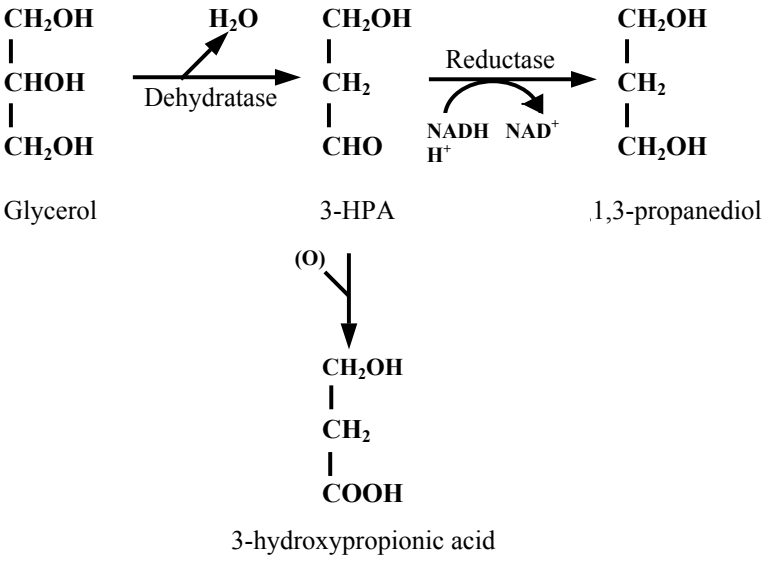


Figure 2. Metabolic pathway for the production and further reduction or oxidation of 3-HPA from glycerol. Adopted from Slininger, Bothast & Smiley (1983).

The production of reuterin (3-HPA) has earlier been reported from *L. brevis* and *L. buchneri* (Schütz & Radler, 1984), *L. collinoides* (Claisse & Lonvaud-Funel, 2000) and *L. coryniformis* (Nakanishi *et al*., 2002; **IV**). Two isofunctional enzymes, glycerol dehydratase and diol dehydratase, catalyse the conversion of glycerol to 3-HPA, but the glycerol dehydratase has greater affinity for glycerol (Sauvageot, 2002a, b). Sauvageot *et al*. (2002a) proposed that three genes (*pdu*CDE), encoding the diol dehydratase of *Lactobacillus collinoides*, are responsible for the first metabolic step in the transformation of glycerol to 3-HPA, *L. collinoides*. This was further supported by biochemical data (Sauvageot *et al.* 2002b). While investigating the presence of diol/glycerol dehydratase genes in *L. coryniformis* Si3 we discovered that Si3 also has a pdu operon containing similar genes as the *L. collinoides* operon (**IV**).

Purification of antifungal low molecular weight compounds

In our work, purification of hydrophobic low molecular weight inhibitory compounds from cell free supernatants of LAB cultures was achieved through a two-step purification procedure including solid phase extraction (SPE) and C_{18}-column high pressure liquid chromatography (HPLC) (Figure 3). The hydrophilic water phase was not further evaluated in this work.

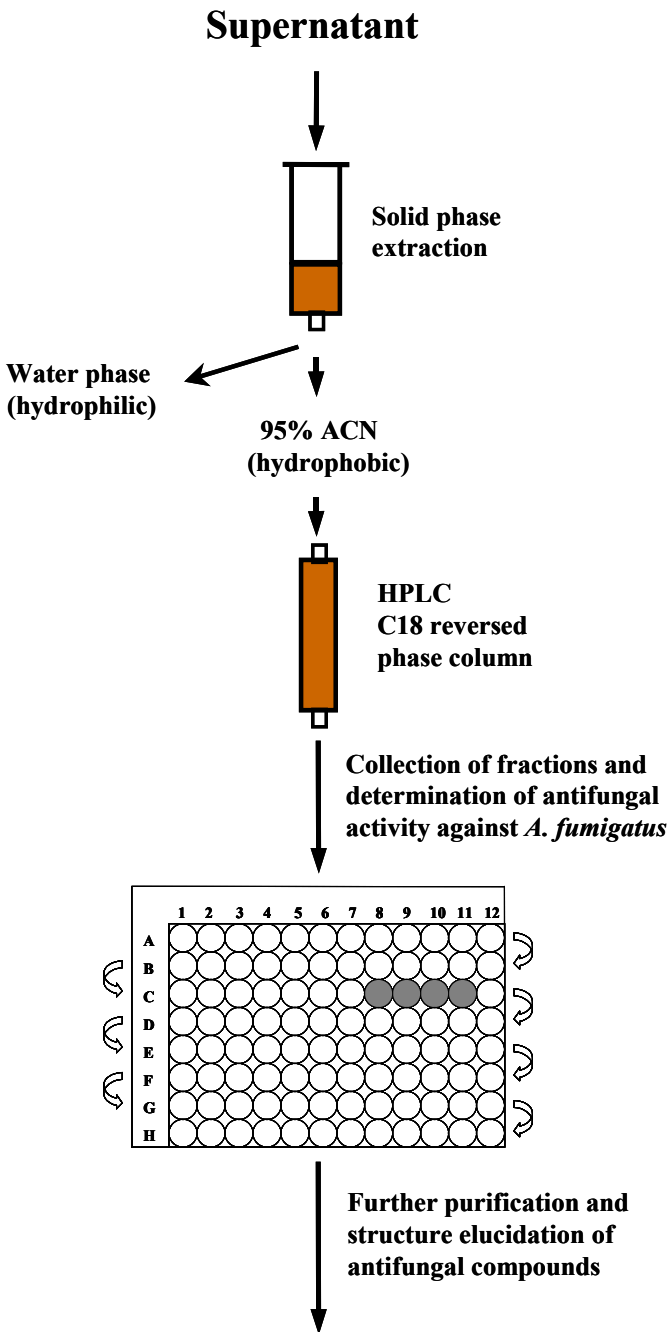


Figure 3. Purification scheme for isolation of antifungal low molecular weight compounds from lactic acid bacteria. Sterile filtered supernatant of LAB is separated on a solid phase extraction column. The hydrophobic fraction is further separated on a C18 reversed phase column and fractions are collected in a 96 well microtiter plate. The antifungal activities of the different fractions are evaluated against the indicator fungus *Aspergillus fumigatus* (**I, III**). (ACN = Acetonitrile)

Fractions with inhibitory activity against the indicator fungus *A. fumigatus* were further purified in a second preparative HPLC run with a porous graphite carbon column. Structures of antifungal compounds isolated during the second HPLC were determined using nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS; **I**, **III**; Ström *et al*. 2002). The bioassay-guided isolation of antifungal compounds was highly reproducible, and patterns of inhibition clearly indicative of the compounds produced. Different species of lactic acid bacteria, gave specific inhibition patterns. This might in the future be developed to a "chemo-taxonomic" fingerprint method for evaluating new antifungal lactic acid bacteria. (Figure 4, **I**, unpublished results).

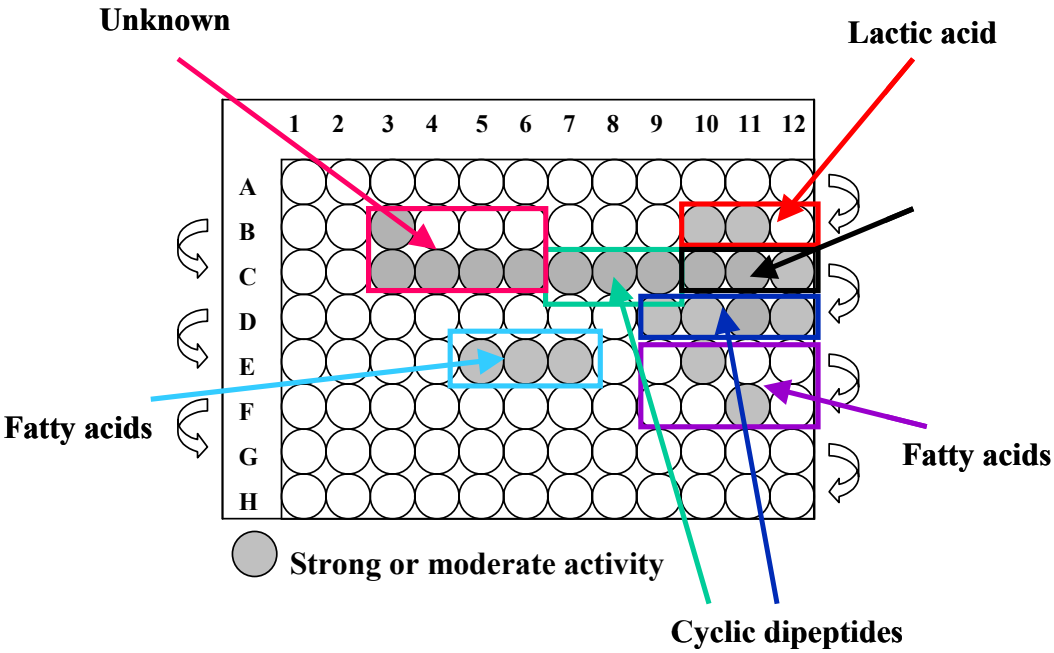


Figure 4. Overview of the antifungal activities found in the microtiter plate well assay after partial purification of LAB supernatants. Several distinct areas with antifungal activity are found corresponding to different low molecular weight compounds. The cyclic dipeptide cyclo(Phe-4-OH-Pro) is found in fractions C10-C12, cyclo(Phe-Pro) and phenyllactic acid in fractions D9-D12 and lactic acid in fractions B10-B12. New antifungal cyclic dipeptides are found in fractions C7-C9. Fatty acids are found in fractions E5-E7 and E9-F9. The remaining wells with antifungal activity contain unknown substances. Arrows indicates movement of fraction collector (**I**, **III**, unpublished results).

Fatty acids

Using the bioassay guided isolation protocol presented in figure 3 we discovered several hydroxylated fatty acids with antifungal activity from *Lactobacillus*

plantarum MiLAB 14 (**III**). Prior to our investigations, no reports on the antifungal activity of hydroxylated fatty acids produced by LAB were available. The fatty acids from MiLAB 14, 3-hydroxydecanoic acid (myrmicacin, 3-HDA), 3-hydroxydocecanoic acid, 3-hydroxytetradecanoic acid and 3-hydroxy-5-cis-dodecenoic acid were isolated from the supernatant (Figure 5).

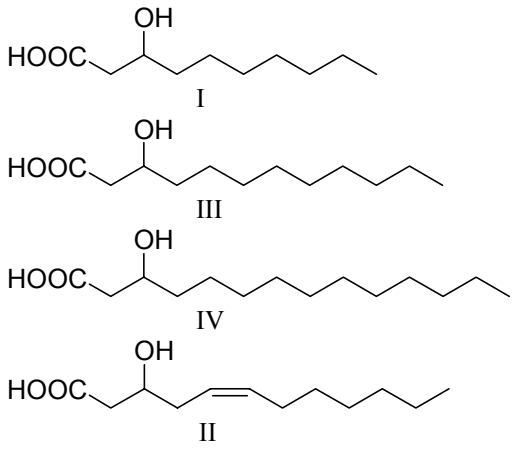


Figure 5. Structure of four fatty acids detected in the supernatant of *L. plantrum* strain MiLAB 14; I, 3-hydroxydecanoic acid (myrmicacin, 3-HDA), II, 3-hydroxydodecanoic acid, III, 3-hydroxytetradecanoic acid, IV, 3-hydroxy-5-cis-dodecenoic acid.

Some lipolytic lactic acid bacteria can under certain conditions produce significant concentration of fatty acids with antimicrobial activity, that also contributes to the sensory quality of fermented foods (Earnshaw, 1992). Rao & Reddy (1984) reported the production of several fatty acids from cultures of lactic acid bacteria in fermented milk. While investigating straight-chained fatty acids, Woolford (1975) found that antimicrobial activity increases with chain length. Caprylic (C_8) acid and longer fatty acids are generally the most effective (except hendecanoic, 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 organics 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 the most effective and exhibited detergent-like properties. We observed that the hydroxylated fatty acid with 12 carbons had the strongest antifungal activity (**III**).

Bergsson *et al*., (2001) investigated the effect of fatty acids and monoglycerides on the growth of *Candida 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 is in agreement with the data of Woolford (1975). Corsetti *et al*. (1998) discovered that a *Lactobacillus sanfrancisco* isolate produced a mixture of organic acids with antimould activity. Caproic (C_6) acid played a key role, but other acids such as 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 (**III**). The minimum inhibitory concentration (MIC) of 3-hydroxydecanoic acid was between 10 and 100 μg ml^{-1}, of 3-hydroxydodecanoic acid between 10 and 50 μg ml^{-1}, and of 3-hydroxytetradecanoic acid between 10 and >100 μg ml^{-1} against moulds and yeasts (**III**). This could be compared with standard antifungal drugs, *e.g.* amphoteracin B that inhibits fungal growth at concentrations in the μg ml^{-1} range (McGinnis & Rinaldi, 1991; Frändberg *et al.* 2000). Yeasts were generally more sensitive to the hydroxylated fatty acids.

Production of hydroxylated fatty acids followed bacterial growth, indicating that they do not result from cell lysis (**III**). The metabolic role of these hydroxylated fatty acids is not clear. Whether or not they are produced as antimicrobial agents was not possible to conclude from the data obtained in this work.

Interestingly, Schildknecht & Koob (1971) isolated β-hydroxydecanoic acid (myrmicacin, β-HDA, 3-HDA) from gland secretions of the South American leaf-cutting ant *Atta sexdens*. It was assumed that β-hydroxydecanoic acid prevents germination and growth of collected seeds and unwanted fungal spores. Later Schildknecht *et al*, (1973) reported that the compounds phenylacetic acid, indolylacetic acid and L-β-hydroxydecanoic acid (myrmicacin) from the metathoric glands of *Atta Sexdens*, besides being antifungal also have auxin activities. These substances might act as growth inhibitors at high concentrations (against contaminating microorganisms and plants) and growth stimulators at low concentrations. The antifungal effect is vaguely documented and the sample used to investigate antimicrobial activity, secrete taken directly from the metathorical glands of another South American leaf-cutting ant *Myrmica rubida*, possibly contained a mixture of organic acids (Maschwitz, Koob & Schildknecht, 1970). The antifungal effect of myrmicacin was also shortly discussed in another publication, but very few data supporting this effect were presented (Schildknecht & Koob, 1971).

Cyclic dipeptides and other low molecular weight inhibitory compounds

Niku-Paavola *et al*. (1999) discovered new types of antimicrobial compounds from the culture filtrate of *Lactobacillus plantarum* VTT E-78076. The active fraction included benzoic acid, 5-methyl-2,4-imidazolidinedione (methylhydantoine), cyclo(glycyl-L-leucyl) (Figure 6c), and tetrahydro-4-hydroxy-4-methyl-2H-pyran-2-one (mevalonolactone). Ström *et al*. (2002), recently reported the production of two cyclic dipeptides cyclo(Phe-Pro) and cyclo(Phe-

OH-Pro) (Figure 6a, 6b) from the supernatant of MiLAB 393. The antimicrobial effect of several different cyclic dipeptides has been investigated (Graz *et al*., 1999; 2001). The present study revealed the possibility that cyclo(Phe-Pro) and cyclo(Phe-OH-Pro) are also produced by strains of *Pediococcus pentosaceus*, *Lactobacillus sakei* and *Lactobacillus coryniformis* (**I**), and thus might be common LAB metabolites. Ström *et al*. (2002), speculate whether these cyclic dipeptides are in fact involved in a quorum sensing mechanism for the bacteria, which has been seen with similar compounds from *Pseudomonas* spp. The cyclic dipeptides have antifungal activity at mg ml^{-1} concentrations, and hence are much less effective than the hydroxylated fatty acids discovered in this work.

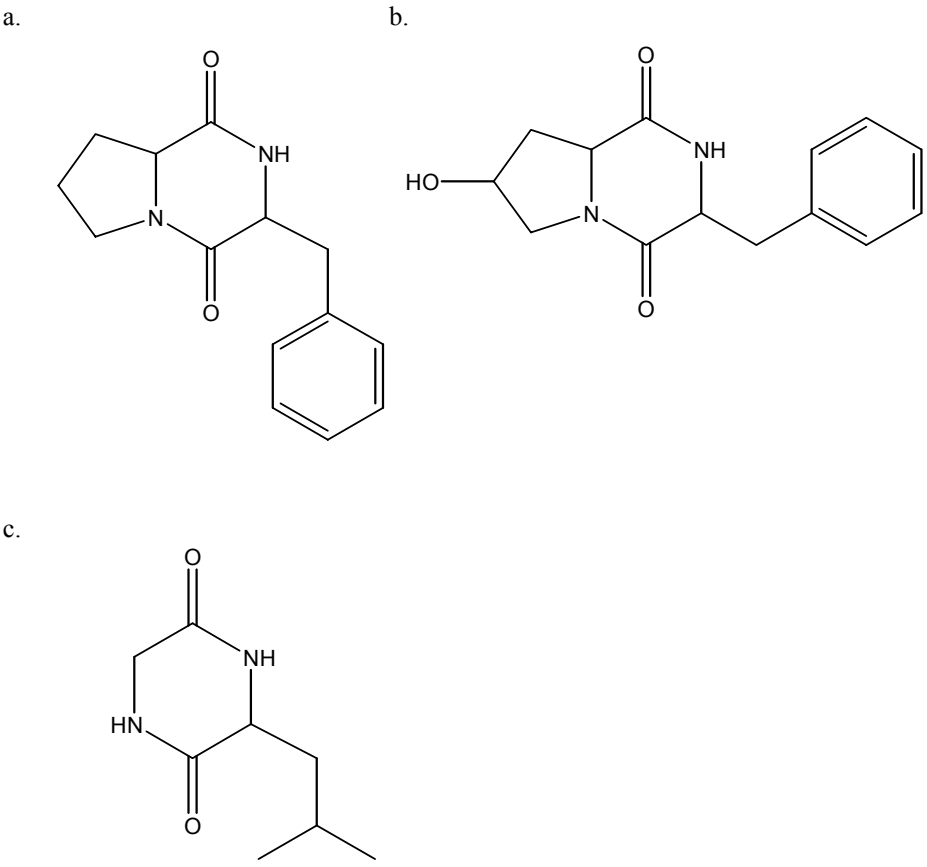
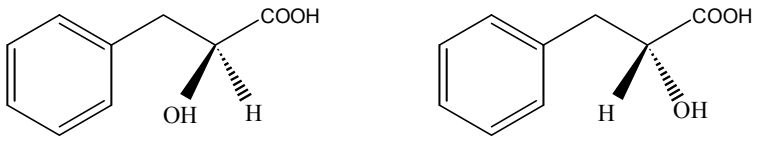


Figure 6. Several cyclic dipeptides with antifungal activity have recently been purified from different strains of LAB. 5 a. cyclo(Phe-Pro) from *Lactobacillus. plantarum* MiLAB 393, *Lactobacillus. coryniformis* strain Si3; 5 b. cyclo(Phe-OH-Pro) from *L. plantarum* MiLAB 393 and *L. coryniformis* strain Si3; 5c, cyclo(Gly-Leu), from *L. plantrum* (Niku-Paavola *et al*., 1999).

We have recently found that there are low concentrations of several cyclic dipeptides present in MRS (broth and agar) and other complex media for growth of lactic acid bacteria. However, these amounts are not themselves high enough to exert antimicrobial activity in the applied bioassays (Unpublished results).

Lavermicocca *et al*. (2000) reported the production of phenyllactic acid and 4-hydroxyphenyllactic acid from *L. plantarum* 21b, which has antifungal activity against several species of filamentous fungi. 3-Phenyllactic acid has also been identified from culture supernatants of *Lactobacillus plantarum* MiLAB 393 (Ström *et al*., 2002, Figure 7) and from *Lactobacillus coryniformis* strain Si3 (**I**). Later studies revealed the possibility that 3-Phenyllactic acid is also produced by strains of *Pediococcus pentosaceus* and *Lactobacillus sakei* (**I**) and thus might be a common LAB metabolite. In common with the cyclic dipeptides, phenyllactic acid is only active at high concentrations against yeasts and moulds. However, they will most certainly act in synergy with other compounds produced by lactic acid bacteria and contribute to the overall antifungal effect.



L-phenyllactic acid D-Phenyllactic acid

Figure 7. Structural form of L and D- phenyllactic acid isolated from *Lactobacillus plantarum* strain Mi393, and *Lactobacillus coryniformis* strain Si3

Summary of the current knowledge of antifungal compounds from LAB

One objective of this work was to give a more complete picture of the complex nature of antifungal activity of LAB. A summary of the antifungal compounds produced by LAB is thus presented in figure 8. Given the limited resources spent to date on investigations of antifungal LAB, it is highly likely that many new antifungal compounds await discovery. It is also obvious that combinations of LAB species or strains that produce different antifungal compounds could be useful as novel biopreservatives. Possible use of antifungal LAB will also depend on whether these organisms and their metabolites will be seen as safe by the regulatory authorities.

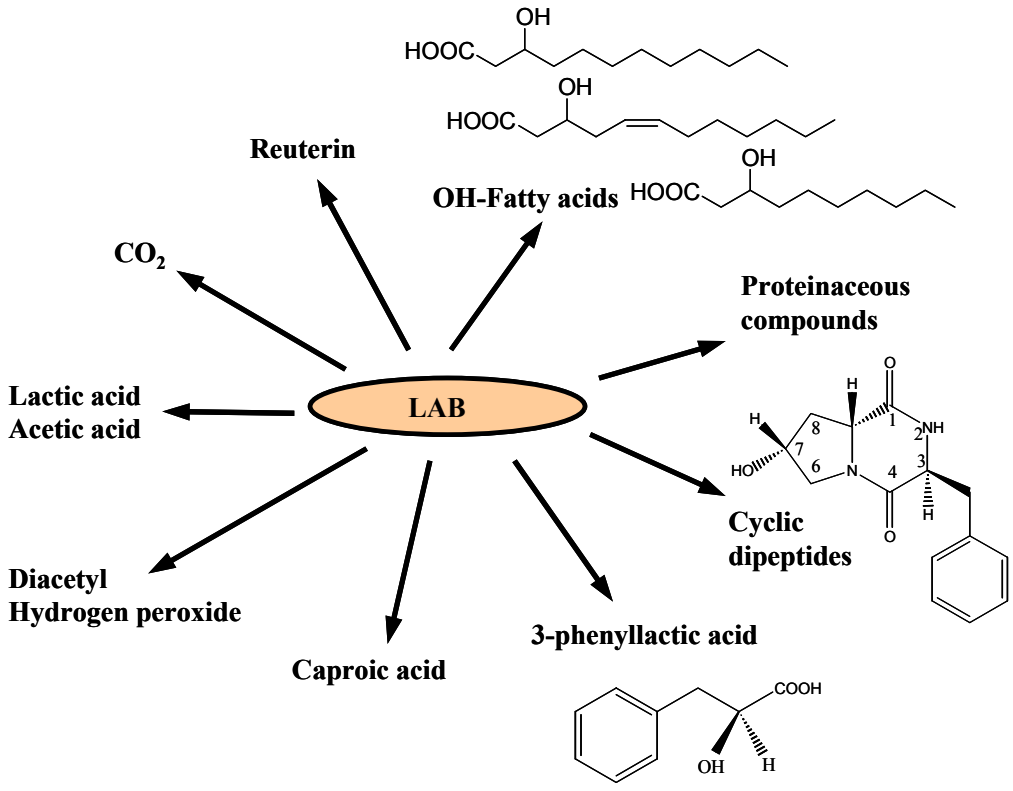


Figure 8. Summary of current knowledge of the complex antifungal activities of lactic acid bacteria.

Concluding remarks

The research field of antifungal lactic acid bacteria is still novel. Most publications on antifungal activity of lactic acid bacteria merely illustrate the activity, but rarely identify active compounds or other reasons for the inhibitory activity. To date, the majority of antifungal substances purified from LAB have been low molecular weight compounds. Only two publications have presented clear evidence for the production of antifungal peptides/proteins. This might be because they are more rare than low molecular weight inhibitory compounds or much more difficult to purify. While reviewing the literature, it appeared that there has to be a large number of very interesting strains, with strong antifungal effect, stored in several culture collections around the world. A re-evaluation of these isolates, with proper controls and purification methods, is needed, to better understand how LAB inhibits fungi.

Studying interactions between lactic acid bacteria and fungi is no simple matter, especially since the bacteria produce fermentation end products that themselves are active to a certain extent, or act synergistically with specific antifungal compounds. In nature, this is of importance for the outcome of microbial interactions. Obviously, each antimicrobial compound produced during lactic acid bacteria fermentation provides an additional hurdle for spoilage organisms to overcome in food or other biotechnological applications.

This work has identified several antifungal compounds, some of which has not previously been reported from lactic acid bacteria. The majority were low molecular weight compounds, *e.g.* hydroxy fatty acids, 3-hydroxypropionaldehyde and cyclic dipeptides, but a proteinaceous compound has also been found. Initially, we believed that it was possible to find one, maybe two active compounds from each isolate, but in fact many more was found. The large number of active compounds probably contributes to a highly complex mode of action.

The results obtained in this study leads to several important questions to be answered by further research; why are these compounds produced, what kind of effect do they really have on the fungus, and are they applicable to food and feed preservation?

In more detail:

- Determine the antimicrobial effect against a broader spectrum of organisms, e.g. bacteria and viruses.
- Investigate the role of cyclic dipeptides and hydroxy fatty acids in the metabolism of lactic acid bacteria.
- Evaluate possible synergistic effects of cyclic dipeptides, phenyllactic acids, and hydroxy fatty acids from antifungal lactic acid.
- Investigate if it is possible to optimise the production of cyclic dipeptides and hydroxy fatty acids, and determine whether other strains of lactic acid bacteria produce similar substances.

- Elucidate molecular response of the fungus when treated with cyclic dipeptides, antifungal peptides, reuterin and the hydroxy fatty acids.
- Investigate if the lactic acid bacteria included in this study can be used for application in food and feed systems.
- Determine the amino acid sequence of the antifungal peptide(s) produced by Lactobacillus coryniformis strain Si3, either through purification of the substance or cloning. There are several interesting aspects to investigate;
    - Similarities to bacteriocins produced by lactic acid bacteria
    - The mode of action of the peptide(s)
    - Gene structure and regulation

This thesis will hopefully inspire other researchers to elucidate the mode of action of antifungal LAB, as well as to develop alternative means of controlling fungal growth in food and feeds.

References

Anderssen, E.L., Diep, D.B., Nes, I.F., Eijsink, V.G.H. & Nissen-Meyer, J. 1998. Antagonistic activity of *Lactobacillus plantarum* C11: Two new two-peptide bacteriocins, plantaricins EF and JK, and the induction factor plantaricin A. *Applied and Environmental Microbiology 64*, 2269-2272.

Andersson, H., Asp, N.-G., Bruce, Å., Roos, S., Wadström, T. & Wold, A.E. 2001. Health effects of probiotics and prebiotics. A literature review on human studies. *Scandinavian Journal of Nutrition 45*, 58-75.

Axelsson, L. (1990). *Lactobacillus reuteri, a member of the gut bacterial flora*. Swedish University of Agricultural Sciences. Uppsala, Sweden.

Axelsson, L. 1998. Lactic acid bacteria: Classification and physiology. In *Lactic Acid Bacteria: Microbiology and functional aspects, 2nd Edition, Revised and Expanded*. Edited by S. Salminen & A. von Wright. pp. 1-72. Marcel Dekker, Inc. New York.

Axelsson, L.T., Chung, T.C., Dobrogosz, W.J. & Lindgren, S.E. 1989. Production of a broad spectrum antimicrobial substance by *Lactobacillus reuteri*. *Microbial Ecology in Health and Disease 2*, 131-136.

Baird-Parker, A.C. 1980. Organic acids. In *Microbial ecology of foods*. Edited by J.H. Silliker. pp. 126-135. Academic press. New York, USA.

Batish, V.K., Grover, S. & Lal, R. 1989. Screening lactic starter cultures for antifungal activity. *Cultured Dairy Products Journal 24*, 21-25.

Batish, V.K., Lal, R. & Grover, S. 1990. Studies on environmental and nutritional factors on production of antifungal substance by *Lactobacillus acidophilus* R. *Food Microbiology 7*, 199-206.

Bergsson, G., Arnfinnsson, J., Steingrimsson, O. & Thormar, H. 2001. In vitro killing of *Candida albicans* by fatty acids and monoglycerides. *Antimicrobial Agents and Chemotherapy 45*, 3209-3212.

Björck, L. 1978. Antibacterial effect of the lactoperoxidase system on psychrotrophic bacteria in milk. *Journal of Dairy Research 45*, 109-118.

Björck, L., Rosen, C., Marshall, V. & Reiter, B. 1975. Antibacterial activity of the lactoperoxidase system in milk against pseudomonads and other gram-negative bacteria. *Applied Microbiology 30*, 199-204.

Bonestroo, M.H., Dewit, J.C., Kusters, B.J.M. & Rombouts, F.M. 1993. Inhibition of the growth of yeasts in fermented salads. *International Journal of Food Microbiology 17*, 311-320.

Brul, S. & Coote, P. 1999. Preservative agents in foods. Mode of action and microbial resistance mechanisms. *International Journal of Food Microbiology 50*, 1-17.

Bullerman, L.B. 1977. Incidence and control of mycotoxin producing molds in domestic and imported cheeses. *Annales de la nutrition et de l'alimentation 31*, 435-46.

Cabo, M.L., Braber, A.F. & Koenraad, P.M. 2002. Apparent antifungal activity of several lactic acid bacteria against *Penicillium discolor* is due to acetic acid in the medium. *Journal of Food Protection 65*, 1309-1316.

Callewaert, R., Holo, H., Devreese, B., Van Beeumen, J., Nees, I. & De Vuyst, L. 1999. Characterization and production of amylovorin L471, a bacteriocin purified from *Lactobacillus amylovorus* DCE 471 by a novel three-step method. *Microbiology 145*, 2559-2568.

Carr, F.J., Chill, D. & Maida, N. 2002. The lactic acid bacteria: a literature survey. *Critical Reviews in Microbiology 28*, 281-370.

Chung, T.C., Axelsson, L., Lindgren, S.E. & Dobrogosz, W.J. 1989. In vitro studies on reuterin synthesis by *Lactobacillus reuteri*. *Microbial Ecology in Health and Disease 2*, 137-144.

Claisse, O. & Lonvaud-Funel, A. 2000. Assimilation of glycerol by a strain of *Lactobacillus collinoides* isolated from cider. *Food Microbiology 17*, 513-519.

Coallier-Ascah, J. & Idziak, E.S. 1985. Interaction between *Streptococcus lactis* and *Aspergillus flavus* on production of aflatoxin. *Applied and Environmental Microbiology 49*, 163-167.

Condon, S. 1987. Responses of lactic acid bacteria to oxygen. *FEMS Microbiology Reviews 46*, 269-280.
Corsetti, A., Gobebetti, M., Rossi, J. & Damiani, P. 1998. Antimould activity of sourdough lactic acid bacteria: Identification of a mixture of organic acids produced by *Lactobacillus sanfrancisco* CB1. *Applied Microbiology and Biotechnology 50*, 253-256.
Davidson, M.P. 2001. Chemical preservatives and natural antimicrobial compounds. In *Food microbiology: Fundamentals and frontiers*. Edited by M.P. Doyle, L.R. Beuchat & T.J. Montville. pp. 593-627. ASM press. Washington, USA.
Davidson, P.M., Post, L.S., Branen, A.L. & McCurdy, A.R. 1983. Naturally occurring and miscellaneous food antimicrobials. In *Antimicrobials in foods*. Edited by P.M. Davidson & A.L. Branen. pp. 385-392. Marcel Dekker Inc. New York, USA.
van Dongen, P.W. & de Groot, A.N. 1995. History of ergot alkaloids from ergotism to ergometrine. *European Journal of Obstetrics and Gynecology and Reproductive Biology 60*, 109-116.
Earnshaw, R.G. 1992. The antimicrobial action of lactic acid bacteria: Natural food preservation systems. In *The lactic acid bacteria in health and disease*. Edited by B.J.B. Wood. pp. 211-232. Elsevier. London, UK.
van Egmond, H.P. 2000. Mycotoxins: Detection, reference materials and regulation. In *Introduction to food- and airborne fungi*. Edited by R.A. Samson, E.S. Hoekstra, J.C. Frisvad & O. Filtenborg. pp. 332-338. Centraalbureau voor Schimmelcultures. Utrecht, The Netherlands.
Eklund, T. 1989. Organic acids and esters. In *Mechanisms of action of food preservation procedures*. Edited by G.W. Gould. pp. 161-200. Elsevier Applied Sciences. New York, USA.
El-Gazzar, F.E., Rusul, G. & Marth, E.H. 1987. Growth and aflatoxin production of *Aspergillus parasiticus* NRRL 2999 in the presence of lactic acid and different initial pH values. *Journal of Food Protection 50*, 940-944.
El-Gendy, S.M. & Marth, E.H. 1981. Growth and aflatoxin production by *Aspergillus parasiticus* in the presence of *Lactobacillus casei*. *Journal of Food Protection 44*, 211-212.
El-Nezami, H., Polychronaki, N., Salminen, S. & Mykkanen, H. 2002. Binding rather than metabolism may explain the interaction of two food-grade *Lactobacillus* strains with zearalenone and its derivative alpha-zearalenol. *Applied and Environmental Microbiology 68*, 3545-3549.
Farkas, J. 2001. Physical methods for food preservation. In *Food microbiology: Fundamentals and frontiers*. Edited by M.P. Doyle, L.R. Beuchat & T.J. Montville. pp. 567-592. ASM press. Washington, USA.
Filtenborg, O., Frisvad, J.C. & Thrane, U. 1996. Moulds in food spoilage. *International Journal of Food Microbiology 33*, 85-102.
Fitzsimmons, N. & Berry, D.R. 1994. Inhibition of *Candida albicans* by *Lactobacillus acidophilus*: Evidence for the involvement of a peroxidase system. *Microbios 80*, 125-133.
Florianowicz, T. 2001. Antifungal activity of some microorganisms against *Penicillium expansum*. *European Food Research and Technology 212*, 282-286.
Freese, E., Sheu, C.W. & Galliers, E. 1973. Function of lipophilic acids as antimicrobial food additives. *Nature 241*, 321-325.
Frändberg, E., Petersson, C., Lundgren, L.N. & Schnürer, J. 2000. *Streptomyces halstedii* K122 produces the antifungal compounds bafilomycin B1 and C1. *Canadian Journal of Microbiology 46*, 753-758.
Gobbetti, M. 1998. The sourdough micro flora: Interactions of lactic acid bacteria and yeasts. *Trends in Food Science and Technology 9*, 267-274.
Gourama, H. 1997. Inhibition of growth and mycotoxin production of *Penicillium* by *Lactobacillus* species. *Lebensmittel-Wissenschaft und-Technologie 30*, 279-283.
Gourama, H. & Bullerman, L.B. 1995. Inhibition of growth and aflatoxin production of *Aspergillus flavus* by *Lactobacillus* Species. *Journal of Food Protection 58*, 1249-1256.
Gourama, H. & Bullerman, L.B. 1997. Anti-aflatoxigenic activity of *Lactobacillus casei pseudoplantarum*. *International Journal of Food Microbiology 34*, 131-143.

Graz, M., Hunt, A., Jamie, H., Milne, P. 1999. Antimicrobial activity of selected cyclic peptides. *Pharmazie 54*, 772-775.

Graz, M., Jamie, H., Versluis, C., Milne, P. 2001. Mechanism of an anti-fungal action of selected cyclic peptides. *Pharmazie 56*, 900-901.

Hammes, W.P., Weiss, N. & Holzapfel, W. 1992. The genera *Lactobacillus* and *Carnobacterium*. In *The Prokaryotes. A handbook on the biology of bacteria volume II*. Edited by A. Balowa, H.G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. pp. 15351594. Springer Verlag. New York.

Haskard, C.A., El-Nezami, H.S., Kankaanpaa, P.E., Salminen, S. & Ahokas, J.T. 2001. Surface binding of aflatoxin B-1 by lactic acid bacteria. *Applied and Environmental Microbiology 67*, 3086-3091.

Hildeng-Hauge, H. (1998). *Amphiphilic alpha-helices in membrane-permeabilizing bacteriocins of lactic acid bacteria*. University of Oslo. Oslo, Norway

Hill, J.E. 1989. Method and inoculant for preserving agricultural products for animal feed. US Patent Application 4,842,871

Hirsch, A., Grinsted, E., Chapman, H.R. & Mattick, A.T.R. 1951. A note on the inhibition of an anaerobic sporeformer in Swiss-type cheese by a nisin-producing *Streptococcus*. *Journal of Dairy Research 18*, 205-206.

Hunter, D.R. & Segel, I.H. 1973. Effect of weak acids on amino acid transport by *Penicillium chrysogenum*: Evidence for a proton or charge gradient as the driving force. *Journal of Bacteriology 113*, 1184-1192.

Jay, J.M. 1982. Antimicrobial properties of diacetyl. *Applied and Environmental Microbiology 44*, 525-532.

Jespersen, L. & Jakobsen, M. 1996. Specific spoilage organisms in breweries and laboratory media for their detection. *International Journal of Food Microbiology 33*, 139-155.

Kabra, J.J. 1983. Medium chain fatty acids and esters. In *Antimicrobials in foods*. Edited by M.P. Davidson & A.L. Branen. pp. 109-140. Marcel Dekker Inc. New York, USA.

Kandler, O. 1983. Carbohydrate metabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek 49*, 209-224.

King, S.W., Fowler, G.G. & Vandenbergh, P.A. 1986. Method for inhibiting fungi. European Patent Application EP 0 221 499 A2

Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A. & Gobetti, M. 2000. Purification and characterization of Novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. *Applied and Environmental Microbiology 66*, 4084-4090.

Lindgren, S.E., and & Dobrogosz, W.J. 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. *FEMS Microbiology Reviews 87*, 149-164.

Loureiro, V. 2000. Spoilage yeasts in foods and beverages: Characterisation and ecology for improved diagnosis and control. *Food Research International 33*, 247-256.

Loureiro, V. & Querol, A. 1999. The prevalence and control of spoilage yeasts in foods and beverages. *Trends in Food Science & Technology 10*, 356-365.

Luchese, R.H. & Harrigan, W.F. 1990. Growth of, and aflatoxin production by *Aspergillus parasiticus* when in the presence of either *Lactococcus lactis* or lactic acid and at different initial pH values. *Journal of Applied Bacteriology 69*, 512-519.

Makanjuola, D.B., Tymon, A. & Springham, D.G. 1992. Some effects of lactic-acid bacteria on laboratory-scale yeast fermentations. *Enzyme and Microbial Technology 14*, 350-357.

de Man, J.C., Rogosa, M. & Sharpe, M.E. 1960. A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology 23*, 130-135.

Maschwitz, U., Koob, K. & Schildknecht, H. 1970. Ein beitrag zur funktion der metathoracaldrüse der amaisen. *Journal of Insect Physiology 16*, 387-404.

Mattila-Sandholm, T., Mättö, J. & Saarela, M. 1999. Lactic acid bacteria with health claims - interactions and interference with gastrointestinal flora. *International Dairy Journal 9*, 25-35.

McGinnis, M.R. & Rinaldi, M.G. 1991. Antifungal drugs: Mechanisms of action, drug resistance, susceptibility testing, and assays of activity in biological fluids. In *Antibiotics in laboratory medicine*. Edited by V. Lorian. pp. 198-257. Williams and Wilkins. Baltimore.

Moll, G., Hildeng-Hauge, H., Nissen-Meyer, J., Nes, I.F., Konings, W.N. & Driessen, A.J. 1998. Mechanistic properties of the two-component bacteriocin lactococcin G. *Journal of Bacteriology 180*, 96-99.

Moon, N.J. 1983. Inhibition of the growth of acid tolerant yeasts by acetate, lactate and propionate, and their synergistic mixtures. *Journal of Applied Bacteriology 55*, 453-460.

Mørtvedt-Abildgaard, C.I., Nissen-Meyer, J., Jelle, B., Grenov, B., Skaugen, M. & Nes, I.F. 1995. Production and pH-dependent bactericidal activity of lactocin S, a lantibiotic from *Lactobacillus sake* L45. *Applied and Environmental Microbiology 61*, 175-179.

Mäyrä-Mäkinen, A.K., Kristianinkatu. A. & Suomalainen, T.V. 1994. A novel microorganism strain, bacterial preparations comprising said strain, and use of saidstrain and preparations for the controlling of yeasts and moulds. European Patent Application 0 576 780 A2

Nakanishi, K., Tokuda, H., Ando, T., Yajima, M., Nakajima, T., Tanaka, O. & Ohmomo, S. 2002. Screening of lactic acid bacteria having the ability to produce reuterin. *Japanese Journal of Lactic Acid Bacteria 13*, 37-45.

Nes, I.F., Diep, D.B., Håvarstein, L.S., Brurberg, M.B., Eijsink, V. & Holo, H. 1996. Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie Van Leeuwenhoek 70*, 113-128.

Nes, I.F. & Holo, H. 2000. Class II antimicrobial peptides from lactic acid bacteria. *Biopolymers 55*, 50-61.

Nielsen, P.V. & de Boer, E. 2000. Food preservatives against fungi. In *Introduction to food- and airborne fungi*. Edited by R.A. Samson, E.S. Hoekstra, J.C. Frisvad & O. Filtenborg. pp. 357-363. Centraalbureau voor Schimmelcultures. Utrecht, The Netherlands.

Niku-Paavola, M.-L., Laitila, A., Mattila-Sandholm, T. & Haikara, A. 1999. New types of antimicrobial compounds produced by *Lactobacillus plantarum*. *Journal of Applied Microbiology 86*, 29-35.

Nilsen, T., Nes, I.F. & Holo, H. 1998. An exported inducer peptide regulates bacteriocin production in *Enterococcus faecium* CTC492. *Journal of Bacteriology 180*, 1848-1854.

Nissen-Meyer, J. & Nes, I.F. 1997. Ribosomally synthesized antimicrobial peptides: their function, structure, biogenesis, and mechanism of action. *Archives of Microbiology 167*, 67-77.

Okkers, D.J., Dicks, L.M.T., Silvester, M., Joubert, J.J. & Odendaal, H.J. 1999. Characterization of pentocin TV35b, a bacteriocin-like peptide isolated from *Lactobacillus pentosus* with a fungistatic effect on *Candida albicans*. *Journal of Applied Microbiology 87*, 726-734.

Packer, S. 1998. Jewish mystical movements and the European ergot epidemics. *Israel Journal of Psychiatry and Related Sciences 35*, 227-241.

Piard, J.C. & Desmazeaud, M. 1991. Inhibiting factors produced by lactic acid bacteria. 1. Oxygen metabolites and catabolism end-products. *Lait 71*, 525-541.

Piard, J.C. & Desmazeaud, M. 1992. Inhibiting factors produced by lactic acid bacteria. 2. Bacteriocins and other antibacterial substances. *Lait 72*, 113-142.

Pitt, J.I., and Hocking, A. D. 1999. Fungi and food spoilage. *Chapman and Hall, New York*

Pohland, A.E. 1993. Mycotoxins in review. *Food Additives and Contaminants 10*, 17-28.

Rao, D.R. & Reddy, J.C. 1984. Effects of lactic fermentation of milk on milk lipids. *Journal of Food Science 49*, 748-750.

Reiss, J. 1976. Prevention of the formation of mycotoxins in whole wheat bread by citric acid and lactic acid. (Mycotoxins in foodstuffs. IX). *Experientia 32*, 168-169.

Rodríguez, J.M., Martinez, M.I., Suarez, A.M., Martinez, J.M. & Hernandez, P.E. 1997. Unsuitability of the MRS medium for the screening of hydrogen peroxide-producing lactic acid bacteria. *Letters in Applied Microbiology 25*, 73-74.

Ross, P.R., Morgan, S. & Hill, C. 2002. Preservation and fermentation: past, present and future. *International Journal of Food Microbiology 79*, 3-16.

Roy, U., Batish, V.K., Grover, S. & Neelakantan, S. 1996. Production of antifungal substance by *Lactococcus lactis* subsp. *lactis* CHD-28.3. *International Journal Food Microbiology 32*, 27-34.

Samson, R.A., Hoekstra, E.S., Frisvad, J.C. & Filtenborg, O. 2000. *Introduction to food- and airborne fungi.* 6th edn. Centraalbureau voor schimmelcultures. Utrecht pp.

Sanglard, D. 2002. Resistance of human fungal pathogens to antifungal drugs. *Current Opinion in Microbiology 5*, 379-385.
Sauvageot, N., Muller, C., Hartke, A., Auffray, Y. & Laplace, J.M. 2002a. Characterisation of the diol dehydratase *pdu* operon of *Lactobacillus collinoides*. *FEMS Microbiology Letters 209*, 69-74.
Sauvageot, N., Pichereau, V., Louarme, L., Hartke, A., Auffray, Y. & Laplace, J.M. 2002b. Purification, characterization and subunits identification of the diol dehydratase of *Lactobacillus collinoides*. *European Journal of Biochemistry 269*, 5731-5737.
Schildknecht, H. & Koob, K. 1971. Myrmicacin, the first insect herbicide. *Angewandte Chemie International Edition 10*, 124-125.
Schildknecht, H., Reed, P.B., Dewitt-Reed, F. & Koob, K. 1973. Auxin activity in the symbiosis of leaf-cutting ants and their fungus. *Insect Biochemistry 3*, 439-442.
Schleifer, K.H. & Ludwig, W. 1995. Phylogeny of the genus *Lactobacillus* and related genera. *Systematic and Applied Microbiology 18*, 461-467.
Schütz, H. & Radler, F. 1984. Anaerobic reduction of glycerol to propanediol-1,3 by *L. brevis* and *L. buchneri*. *Systematic and Applied Microbiology 5*, 169-178.
Slininger, P.J., Bothast, R.J. & Smiley, K.L. 1983. Production of 3-hydroxypropionaldehyde from glycerol. *Applied and Environmental Microbiology 46*, 62-67.
Sobolov, M. & Smiley, K.L. 1960. Metabolism of glycerol by an acrolein-forming *Lactobacillus*. *Journal of Bacteriology 79*, 261-266.
Stiles, J., Penkar, S., Plockova, N., Chumchalova, J. & Bullerman, L.B. 2002. Antifungal activity of sodium acetate and *Lactobacillus rhamnosus*. *Journal of Food Protection 65*, 1188-1191.
Stiles, M.E. 1996. Biopreservation by lactic acid bacteria. *Antonie Van Leeuwenhoek 70*, 331-345.
Ström, K., Sjögren, J., Broberg, A. & Schnürer, J. 2002. *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo($_L$-Phe-$_L$-Pro) and cyclo($_L$-Phe-trans-4-OH-$_L$-Pro) and phenyl lactic acid. *Applied and Environmental Microbiology 68*, 4322-4327.
Suzuki, I., Nomura, M. & Morichi, T. 1991. Isolation of lactic acid bacteria which suppress mold growth and show antifungal action. *Milchwissenschaft 46*, 635-639.
Talarico, T.L., Casas, I.A., Chung, T.C. & Dobrogosz, W.J. 1988. Production and isolation of reuterin, a growth inhibitor produced by *Lactobacillus reuteri*. *Antimicrobial Agents and Chemotherapy 32*, 1854-1858.
Talarico, T.L. & Dobrogosz, W.J. 1990. Purification and characterization of glycerol dehydratase from *Lactobacillus reuteri*. *Applied and Environmental Microbiology 56*, 1195-1197.
Vandenbergh, P.A. & King, S.W. 1988. Process for producing novel yeast and mold inhibiting products. EP 0,302,300,B1
Vandenbergh, P.A. & Kunka, B.S. 1989. Antifungal product. United States Patent Number 4,877,615
Viljoen, B.C. 2001. The interaction between yeasts and bacteria in dairy environments. *International Journal of Food Microbiology 69*, 37-44.
Wiseman, D.W. & Marth, E.H. 1981. Growth and aflatoxin production by *Aspergillus parasiticus* when in the presence of *Streptococcus lactis*. *Mycopathologia 73*, 49-56.
Woolford, M.K. 1975. Microbiological screening of the straight chain fatty acids (C1-C12) as potential silage additives. *Journal of the Science of Food and Agriculture 26*, 219-228.
Woolford, M.K. 1984b. The antimicrobial spectra of some salts of organic acids and glutaraldehyde in respect to their potential as silage additives. *Grass and Forage Science 39*, 53-57.
Woolford, M.K. 1984a. The antimicrobial spectra of organic compounds with respect to their potential as hay preservatives. *Grass and Forage Science 39*, 75-79.

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