Antifungal Properties of Dairy Propionibacteria

Helena Lind
Faculty of Natural Resources and Agricultural Sciences
Department of Microbiology
Uppsala

Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2010

Acta Universitatis agriculturae Sueciae
2010:44

ISSN 1652-6880
ISBN 978-91-576-7457-9

Print: SLU Service/Repro, Uppsala 2010

Antifungal Properties of Dairy Propionibacteria

Abstract

Today global agriculture is facing major challenges. The world's population is growing rapidly and we need to meet the increasing need for food with new strategies, including environmentally sustainable practices. Production must be enhanced with optimised resource utilisation and improved storage and distribution techniques. In addition, consumer demands for a reduction in artificial preservatives require innovative research thinking.

The use of microorganisms as biopreservatives has many advantages. They often originate from environments similar to those where they will be used, making their presence natural. Bacteria such as lactic acid bacteria and dairy propionibacteria produce weak organic acids with good preserving capacity and several other antimicrobial compounds. Dairy propionibacteria possess additional advantageous features such as high production of vitamin B_{12} and folic acid. Some strains also produce exopolysaccharides, useful for texture enhancement in dairy products.

This thesis focuses on the antifungal properties of dairy propionibacteria. An important part of their antifungal capacity is due to the production of organic acids. The effect of these acids at different pH and different concentrations against eight spoilage fungi was investigated. A pH-dependent growth inhibition effect was detected, with *Penicillium roqueforti* being the most sensitive fungal species. In addition, dairy propionibacteria produce a number of other compounds with antifungal activity. Identification of 3-phenyl lactic acid and several antifungal peptides present in the growth medium was made after separation with a solid phase extraction column and HPLC. The antifungal activity was monitored by screening against *Aspergillus fumigatus*. Strong enhancement of the antifungal activity from dairy propionibacteria in combination with glycerol was demonstrated. Addition of 500 mM glycerol to the upper layer in an overlay assay reduced the growth of some spoilage fungi completely. An antifungal strain of *Propionibacterium jensenii* was combined with *Lactobacillus fermentum* in a biopreserving culture used in moist grain preservation. The addition of glycerol to the system enhanced production of propionic acid in some cases, and an earlier decline in pH was detected. However, this combination of bacteria was not able to reduce the growth of *P. roqueforti*.

Keywords: dairy propionibacteria, organic acid, antifungal, glycerol, biopreservation

Author's address: Helena Lind, SLU, Uppsala BioCenter, Department of Microbiology, P.O. Box 7025, SE-750 07 Uppsala, Sweden
E-mail: Helena.Lind@mikrob.slu.se

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Helena Lind**, Hans Jonsson and Johan Schnürer (2005). Antifungal effect of dairy propionibacteria – contribution of organic acids. *International Journal of Food Microbiology* 98, 157-165.

II **Helena Lind**, Jörgen Sjögren, Suresh Gohil, Lennart Kenne, Johan Schnürer and Anders Broberg (2007). Antifungal compounds from cultures of dairy propionibacteria type strains. *FEMS Microbiology Letters* 271, 310-315.

III **Helena Lind**, Anders Broberg, Karin Jacobsson, Hans Jonsson and Johan Schnürer. Glycerol enhances the antifungal activity of dairy propionibacteria (submitted).

IV **Helena Lind**, Malin Larsson, Hans Jonsson, Johan Schnürer and Karin Jacobsson. The use of *Propionibacterium jensenii* and lactic acid bacteria for the preservation of moist grain in the presence of glycerol (manuscript).

The contribution of Helena Lind to the papers included in this thesis was as follows:

I Took part in planning the study. Performed all laboratory work. Main writer of the manuscript.

II Took part in planning and performing the work. Shared responsibility for writing the manuscript.

III Took part in planning the study. Performed all laboratory work. Main writer of the manuscript.

IV Took part in planning the study. Performed main part of the laboratory work. Took part in writing the manuscript.

Abbreviations

LAB	Lactic acid bacteria
hGH	Human growth hormone
ATP	Adenosine-5'-triphosphate
SL	Sodium lactate medium
MRS	de Man Rogosa Sharp medium
MRS-ac	MRS medium without acetate
NMR	Nuclear magnetic resonance
PCR	Polymerase chain reaction
EU	European Union
EFSA	European Food Safety Authority
QPS	Qualified Presumption of Safety
CFU	Colony forming unit

1 Introduction

Microorganisms have always played an important role in our lives, sometimes in a good way. The ancient process of spontaneous fermentation has created a tasty way to preserve food and feed and give us the possibility to store food and feed throughout the year. Lactic acid bacteria (LAB) can provide us with a variety of flavoursome foods such as cheese, yoghurt, pickled vegetables and fermented sausage (Hansen, 2002). Bacteria can also be used for the manufacture of solvents, vitamins and enzymes (Demain & Adrio, 2008). The ability to inhibit other microorganisms expands the usefulness of some bacteria to include prevention of pathogens in the intestine of slaughter animals (Younts-Dahl *et al.*, 2004).

Yeasts are used daily for the production of bread and beverages, while microorganisms such as moulds have also shown a spectrum of useful applications. The most famous example must be the Nobel prize-winning discovery by sir Alexander Fleming in 1928 that the mould *Penicillium* was able to inhibit growth of a bacterial culture by the production of an antibiotic compound (Brown, 2004). More recent applications are the production of quorn, a nutritional meat substitute made from the mycelium of the mould *Fusarium venenatum* (Denny *et al.*, 2008).

Unfortunately, microorganisms can have various negative effects. Bacterial infections cause conditions such as otitis, cystitis and diarrhoea (Danielsson & Kjellander, 1993). Viruses can cause childhood diseases such as chicken pox and measles, but also severe illnesses such as hepatitis and aids (Danielsson & Kjellander, 1993). Yeast infections of human mucous membranes are rather common, while filamentous fungi are more seldom involved in human diseases (Danielsson & Kjellander, 1993). On the other hand, in spoilage of food and feed, filamentous fungi are well represented. At suitable temperature and level of water availability, these fungi can quickly spoil large quantities of edibles. Their growth may lead to spoilage

both by unpleasant smell, taste and appearance and by mycotoxins. Mycotoxins are secondary metabolites produced by certain filamentous fungi and many of them are highly toxic (Magan & Aldred, 2007)

The increasingly critical attitude of consumers to the use of large amounts of chemicals as preservatives has prompted a search for a simple, non-toxic and user-friendly alternative. One important example is the use of microorganisms as biological preservatives, also called biopreservation/biocontrol (Ross *et al.*, 2002).

During growth of certain types of bacteria, organic acids, *e.g.* lactic, acetic and propionic acid are produced. These are effective in decreasing pH, which can be an effective way of inhibiting other microorganisms (Adams & Moss, 2000).

There are several species of bacteria and yeasts that can act as preservative agents, but the focus in this thesis is on dairy propionibacteria. Their best known application so far is probably as starter cultures in Swiss type cheese, to give that special nutty flavour and characteristic holes. Another interesting application is the use of dairy propionibacteria as natural preservatives in food and feed. They have been applied as protective cultures in bread (Soumalainen & Mäyrä-Mäkinen, 1999), dairy products (Ekinci & Gurel, 2008) and animal feed in the form of maize silage (Rowghani *et al.*, 2008), but their use in grain preservation is limited today.

Since dairy propionibacteria have been safely used as starter cultures for decades, their possible use as biocontrol microorganisms should not meet any major obstacles in legislation or from a consumer safety perspective (Meile *et al.*, 2008).

1.1 Aims

This thesis investigated the hypothesis that dairy propionibacteria can be used as natural preservatives/biocontrol organisms in feed storage systems. Specific objectives were to:

- Investigate the inhibitory effect of organic acids produced by dairy propionibacteria.
- Compare growth and antifungal effects of dairy propionibacteria on different substrates.
- Identify antifungal compounds produced by dairy propionibacteria.
- Study possible improvement of the antifungal effect of dairy propionibacteria in combination with glycerol.
- Evaluate dairy propionibacteria as part of a biopreservation culture during storage of moist grain.

2 Agriculture in Change

Due to the great Swedish population explosion, starting in the beginning of the nineteenth century, the demands on agriculture to produce enough food increased heavily. However contrary to common belief, it was not industrialisation or the introduction of artificial fertilisers that saved Sweden from severe famine. Swedish farmers were able to meet the challenge, or at least to avoid the worst case scenario, by changes such as a shift to pasture management (Einarsson, 1996). Previously the winter feed (hay and dried leaves) came exclusively from natural meadows and trees. Thanks to Carl Linnaeus the use of leguminous plants revolutionised the circulation of plant nutrients in arable land (Einarsson, 1996). Legumes possess the ability to utilise nitrogen from the air for their growth, as well as enriching the soil *e.g.* for a following cereal crop. Suddenly farmers had an increased yield of animal feed and could also cultivate their fields continuously, avoiding the need for fallow every second or third year.

It was not until after World War II, that artificial fertilisers and pesticides had their great breakthrough (Jonsson, 1994).

2.1 From Small Farming to Big Industries

The introduction of artificial fertilisers and pesticides increased productivity, but another requirement was the driving force for continuing agricultural development. As in most other production areas, the main aim of the industrialisation of agriculture was to decrease the use of human labour. The introduction of chemical production inputs such as artificial fertilisers and pesticides liberated farmers from the need to balance the recirculation of nutrients in the 1930s and 1940s (Einarsson, 1996). Instead of being dependent on a complete functioning production chain, with animals producing manure that could fertilise the fields or the use of leguminous

plants, farmers were now able to focus on one type of activity, either animal breeding or crop production. Animal feed could be bought from crop farmers and fertilisers were commercially available. Unfortunately this development came with a predetermined consequence: since new techniques and chemical additives were labour-saving, the price of agricultural produce fell, so farmers were more or less forced to keep up with developments.

2.2 Sustainable Development

The initial reason for the rapid change in farming was the same as the reason for all industrial evolution: The convenience and labour-saving aspects of a change from muscle work to large-scale mechanised production (Einarsson, 1996). However, after 50 years of increasing use of chemical fertilisers and pesticides, more and more people are realising that this trend cannot continue. The total number of hectare-doses of chemical pesticides sold in Sweden 2008 was 5.1 millions, a 16 % increase compared with 2007. In relation to the total area of arable land, the number of hectare-doses sold during the 1990s increased from 1.10 to 1.69. A few years of a decreasing trend followed, but in 2003 there was a considerable shift to an increase (Bengtsson & Persson, 2009). One explanation could be the more intense use of arable land and decrease in set-aside fields. Another could be a lowered acceptance threshold for fungal deterioration due to expected rise in grain prices. In any case, this trend for an increase is worrying and needs to be taken seriously.

Considering the fact that 80% of the Swedish arable land area used for crop production is employed for animal feed production, a change in our consumption pattern may be needed for a sustainable future (Rudquist & Lundgren, 1996). A simple mathematical exercise shows the imbalance in today's allocation of resources. The same area that is needed for production of sufficient animal protein to feed one man for 80 days will produce sufficient vegetable protein (soybean) to feed for the same man for 2 200 days (Käck-Brolund, 1991)!

Another aspect of the non-sustainable way we are handling resources is the pollution we are producing from all transport associated with food and feed production. The proportion of transport costs included in our daily food prices is continually increasing, surely at the expense of the environment as well.

Recently, Rockström *et al.* (2009) proposed planetary boundaries for nine processes that urgently need attention. One of those where we have already exceeded the acceptable limit is the nitrogen cycle. Others are the change in land use, where the conversion of unexploited land into crop land must be slowed down, and of course all chemical pollution. All these processes are dependent on the agricultural industry, in one way or another. The conversion of land is obvious and chemical pollution is a result of many kinds of processes, where greenhouse gas emissions from transportation are only a small part. The contribution of nitrogen released from modern agriculture is significant. The manufacture of fertilisers for food production and the cultivation of leguminous plants has become an important and major cause of the exceeded pollution limit of nitrogen gases. Around 120 million tonnes of nitrogen gas from the atmosphere are converted into reactive forms every year, primarily by these processes (Rockström *et al.*, 2009).

To summarise, we need to change the way we manage our natural resources and simply how we treat our planet, if we are going to have a sustainable ecosystem for coming generations. The claim that a part of the solution is to introduce organic agriculture is controversial. In organic agriculture, no inorganic fertilisers or chemical pesticides are allowed. An advantage of this is of course the limitation of pollution. On the other hand, organic agriculture results in lower crop yields, so in order to feed the same amount of people a larger land area would need to be exploited. The question of conversion of world agriculture to organic management is dividing experts and interest groups into two different camps. One of the most heated conflicts is the question of whether the production of commercial fertilisers is increasing greenhouse gas emissions (Andersson *et al.*, 2009). A relevant question in this context is how the excess nitrogen from animal rearing and from urban living (sewage waters) can be used as fertilisers in crop production. It is important to find a sustainable agricultural system with nutrients and energy circulating to a higher degree than today. In addition, we might need to supplement our diet with more from the vegetable kingdom in order to meet the demand for food from the world's growing population.

2.3 Food and Feed

Humans have developed an advanced system for the production, handling and preparation of food. We have legislation controlling every step in the production process. In developed countries a substantial proportion of all

food produced is thrown away, either already in the supermarket or by consumers, due to too strict recommendations from authorities, combined with ignorance of keeping properties (SIWI, 2008).

Our farm animals live a simpler life. Feed and water are supplied by the farmer, hopefully nutritionally balanced. Animal feed production is also surrounded with many restrictions, and places great responsibility on the producer regarding safety and nutritional requirements.

A sustainable future in food and feed production and consumption requires more common knowledge about food safekeeping and responsibility to be taken for the consequences of today's thriftless way of living.

2.3.1 Production and Consumption

Approximately 3% of the paid workforce in Sweden was working in agriculture or the food industry at the end of 2009 (Rahm, 2010). That means that only 90 000 people are responsible for national food and feed production for Sweden's 9 million people. The demands on food do not simply concern volume and taste anymore. Today we also want to know that our food is being produced without a negative influence on animal or plant diversity, on the health of our seas or on the ozone layer. It also needs to be free from all sorts of toxins or additives liable to cause illness.

In feed production, the expectations are more or less the same, even if the demands are not set by consumers. We need to increase the world's food and feed production, but not at the expense of the future of our planet. One way to meet this need is by utilising existing resources more efficiently. Far too great a proportion of all food and feed is thrown away today. Better planning and stronger awareness would lead to a change in this behaviour. The control of feed utilisation is dependent on limiting spillage and ensuring reliable storage.

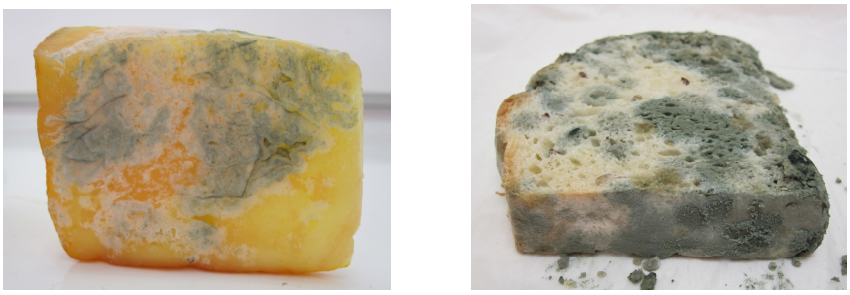

Figure 1. Hard cheese inoculated with *P. roqueforti* and bread naturally deteriorated by *Penicillium* moulds.

2.3.2 Storage and Deterioration

During storage several things can occur that make the product inedible. Wet food can dry out and fatty products can become rancid. However, the major issue for deteriorated food and feed is spoilage by microorganisms (illustrated in Fig. 1).

No food or feed is sterile at the consumer stage. Spoilage microorganisms come either from the raw material or are accidentally introduced at some stage of the production process. Many microorganisms, unintentionally present in the product, are harmless and do not cause any problems by their presence. The trouble starts if detrimental microorganisms are able to grow. The most important regulatory factors are access to nutrients, adequate water activity, suitable pH and suitable temperature (Adams & Moss, 2000). In many food products, the presence of antimicrobial constituents will also restrict growth. Spices and pigments can both enhance the organoleptic experience and prevent growth of spoilage microorganisms (Adams & Moss, 2000).

In order to enhance shelf-life we need to make sure that one or more of the critical factors are limited. The access to nutrients is difficult to restrict if the products are to remain suitable as food or feed. Water activity, pH and temperature are more easily controlled factors. For food, we developed a reliable temperature control system with the introduction of the refrigerator. Storage of feed relies on the control of other factors, since temperature control for such large-scale storage facilities is impossible. The most common way of preserving feed grain in Sweden today is by hot air drying using fossil fuel and electricity (Jonsson & Pettersson, 1999). This results in sufficiently low water activity to ensure safe storage. However, with increasing energy prices and demands for more environmental friendly

treatment, the interest in alternative methods, such as biopreservation, is increasing.

3 Microbial Technology

The number of applications for microbial technology is constantly increasing. The general opinion that microorganisms are mostly of a malign nature would probably change if only a fraction of all possible applications became more commonly known.

Microorganisms can produce several useful products apart from the everyday bread, cheese, yoghurt and beverages. In the 1950s a polysaccharide with valuable properties, produced by the bacterium *Xanthomonas campestris,* was found (Glazer & Nikaido, 2007). The polysaccharide xanthan gum is now used as an additive in a variety of foods, feeds, pharmaceuticals and cosmetics. The structure of xanthan gum makes it useful as an emulsifier and freeze-thaw stabiliser. Another useful substance produced by microorganisms is ethanol. Yeasts such as *Saccharomyces* species and bacteria such as *Clostridium* and *Thermoanaerobium* are known to produce ethanol (Glazer & Nikaido, 2007). Production of ethanol is of course profitable for the alcoholic beverage industry, but the emerging search for an unlimited source of petrochemical-independent transport fuel is making ethanol production even more interesting.

Genetic modification of microorganisms has opened up an even broader range of possible applications. The use of genetically modified microorganisms for the pharmaceutical industry is probably not the most controversial use. Production of insulin and human growth hormone (hGH) from genetically engineered *Escherichia coli* has revolutionised medical treatment of patients with diabetes and growth disorders (Glazer & Nikaido, 2007). However, when it comes to the transfer of microbial genes into plants to achieve resistance to cold, heat, salt or pesticides, the public response in Europe is usually not so accepting.

Another area where the activities of microorganisms can be very useful is in the growing problem of pollution of waters and soil. In waste water

treatment plants, a complex community of organisms (bacteria, fungi, algae, protozoa, *etc.*) is being used to reduce pathogens as well as organic compounds in the water (Glazer & Nikaido, 2007). The definition of bioremediation is: "a spontaneous or managed process in which biological (especially microbiological) catalysis acts on pollutants and thereby remedies or eliminates environmental contamination" (Glazer & Nikaido, 2007). Applications can be found in contaminated soils, ground water or oily sludge from petroleum refineries.

3.1 Food and Feed Improvement by Fermentation

Microorganisms growing anaerobically can enhance the quality and improve the shelf-life of a food or feed product by a fermentation process. Fermentation is an anaerobic process with internally balanced redox reactions (Buckel, 1999), often resulting in acid production, giving rise to improved stability. Most fermentations are initiated by bacteria but there are also products said to be fermented by fungi, sometimes in an aerobic environment, although the usage of the term fermentation in that case is by definition incorrect.

If the microorganism is a yeast, the raw material is often rich in carbohydrates (Adams & Moss, 2000). Examples of yeast-fermented food are beer, wine and bread. Some fermented foods are produced by inoculation of filamentous fungi (moulds). In eastern countries such as Indonesia and China, the consumption of fermented foods made with moulds is widespread. Tempeh is a generic term for cereals or beans fermented by the growth of a filamentous fungus, but usually refers to soybean fermented by *Rhizopus* species, preferably *Rhizopus oligosporus* (Nout & Kiers, 2005). However, cereal grains have also been successfully used as raw material (Feng *et al.*, 2005). Another product created by mould fermentation is soy sauce. The characteristics of soy sauce are due in part to species of *Aspergillus* (*A. oryzae*, *A. sojae* and *A. niger*), but other microorganisms are also required for a complete soy sauce product. Alcoholic fermentation by the yeast *Zygosaccharomyces rouxii* and lactic acid fermentation by the LAB *Tetragenococcus halophila* are also used to produce soy sauce with characteristic taste and flavour (Nout, 2007).

The largest number of fermented products relies on LAB fermentation. They cover most food product areas with everything from yoghurt, kefir and cheese to fermented vegetables such as sauerkraut, olives and pickles, sausages such as salami and even fermented fish, the foundation of many Asian sauces and pastes (Adams & Moss, 2000).

3.2 Biopreservation

Biopreservation and fermentation processes share several properties. Both concepts involve microorganisms and their ability to produce substances enhancing the quality of the product. In the fermentation process, the focus is on producing a pleasant flavour, odour and texture, and at the same time prolonging shelf-life. Although similar activities can occur in biopreservation, the focus in this work is on the preserving quality.

Biopreservation is performed with microorganisms naturally occurring in the product, or with microorganisms closely related to those naturally occurring, without the intention of interfering with the organoleptic experience.

Traditionally, microorganisms producing a spectrum of antimicrobial compounds are used in biopreservation, with LAB being particularly useful. Due to the wide range of potential applications, and the long history of safe use in both food and feed, there has been an interest in identifying active metabolites from LAB. In addition to the obvious effect of the lactic and acetic acid produced, a variety of compounds able to inhibit other microorganisms have been found. Small peptides and 3-phenyllactic acid (Ghalfi *et al.*, 2010; Ström *et al.*, 2002), proteinaceous compounds (Magnusson & Schnürer, 2001) and bacteriocins (Hata *et al.*, 2010; Tiwari & Srivastava, 2008; Kumari & Prakash Garg, 2007) have all displayed antimicrobial activity.

3.2.1 Mode of Action

The mode of action of all these different compounds is not yet fully understood. Regarding the effect of organic acids on spoilage organisms, the inhibitory capacity is related to the pH value. In dilute solutions, pH is directly related to the concentration of hydrogen ions. The release of hydrogen ions from an acid is dependent on the strength of the acid. Acids produced from fermenting bacteria are weak organic acids (acetic, lactic and propionic acid). These types of acids only partly release their hydrogen ions in the pH range of foods (Stratford, 1999), *i.e.* they are partially dissociated. In solution, an equilibrium between dissociated and undissociated molecules of weak acids is formed:

$$[HA] \rightleftharpoons [H^+] + [A^-]$$

where H is the proton and A is the acid.

This equilibrium is pH-dependent; high pH leads to higher concentration of the dissociated form, while low pH increases the concentration of the undissociated form. At a certain pH, each weak acid exists with equal proportions of dissociated and undissociated molecules. This pH value corresponds to the pK_a value of the acid. Using the Henderson-Hasselbach equation, the concentration of dissociated (or undissociated) molecules of an acid at a certain pH can be calculated.

$$pH = pK_a + \log \frac{[A^-]}{[HA]} \qquad \text{(Henderson-Hasselbach equation)}$$

The ability to remain in the undissociated state is an essential property giving weak acids the potential to inhibit microbial growth. A strong acid is completely dissociated in the range of pH of most foods. This makes them more efficient in lowering the surrounding pH, but with less specific effect on the target organism. For the weak acids, the undissociated molecules represent the potent growth inhibitory capacity. Since the undissociated molecules are dominant at pH values below the pK_a and rapidly decrease in concentration above the pK_a, a weak acid with a high pK_a value is the most effective at relatively high pH.

The plasma membrane of most microorganisms restricts penetration by charged molecules. However, undissociated molecules can easily diffuse, at least if they are lipophilic (Stratford, 1999). Inside, the microorganisms aim for a pH near neutral. This is necessary to protect cell structural proteins, enzymes, nucleic acids and phospholipids (Davidson, 2001). When an undissociated acid molecule enters the neutral, intracellular environment, the molecule will dissociate into the charged form; one proton and one negatively charged molecule. This acidifies the microbial cell and the endeavour to maintain neutral pH leads to active transport of unwanted molecules. The membrane-bound H^+-ATPase can pump out the protons, but this requires energy in the form of adenosine-5'-triphosphate (ATP). Already at this stage growth will be weakened due to the loss of energy. If the H^+-ATPase pump is unable to restore the internal pH, vital structures and functions inside the microbial cell will be destroyed, eventually leading to cell collapse (Stratford, 1999).

The target organisms are of course very different, which makes them respond to weak acids in different ways. The action of lactic acid, and probably other organic acids, is also believed to act through some other

mechanism, since no correlation between decrease in internal pH and degree of lactic acid inhibition has been observed (Freese *et al.*, 1973).

Bacteriocins produced by LAB lead to the formation of pores in the membrane of the target organisms, causing leakage and destruction of the transmembrane potential (Todorov, 2009; Moll *et al.*, 1996). The bacteriocin dysgalacticin also disrupts the membrane and, in addition, inhibits the glucose uptake (Swe *et al.*, 2009).

4 Dairy Propionibacteria

The scientists E. Freudenreich and O. Orla-Jensen isolated the first propionic acid bacterium from Emmental cheese in 1906 (Jan *et al.*, 2007). In early studies the propionic acid bacteria were exclusively isolated from cheese, milk and dairy products. It is now known that their natural habitat can also be the digestive tract of ruminants (Rinta-Koski *et al.*, 2001; Jarvis *et al.*, 1998). Refined isolation techniques have made it possible to isolate dairy propionibacteria from soil and silage too (Rossi *et al.*, 1999).

The cutaneous propionibacteria, included in the genus *Propionibacterium* in the 1970s, are distinguished from dairy propionibacteria (Vorobjeva, 1999). As the name suggests they are found on human skin, but also in wounds, bone marrow, stomach contents and blood. Originally the cutaneous propionibacteria were placed in the genus *Corynebacterium*, but the transfer was motivated by the fact that they are anaerobes (corynebacteria are mostly aerobic), they produce propionic acid as the main metabolite and their cell wall composition is more similar to dairy propionibacteria than to corynebacteria (Vorobjeva, 1999). However, there are several characteristics separating the two branches of propionibacteria (Table 1). Dairy propionibacteria are also called classic propionibacteria, as they were the first to be included in the genus *Propionibacterium*. This genus belongs to the family *Propionibacteriacea,* a subclass of Actinobacteria, together with genera such as *Luteococcus, Friedmanniella, Tessaracoccus* and *Propionimicrobium*. The phylogenetic relationship among propionibacteria differs from the classification into dairy and cutaneous species. *Propionibacterium freudenreichii* is phylogenetically closest to the only classical species not isolated from dairy products, i.e. *P. cyclohexanicum*. The other classical species are clustered together with cutaneous species such as *P. acnes, P.avidum* and *P.granulosum* in a phylogenetic tree based on 16S rDNA sequences (Stackebrandt *et al.*, 2006).

Table 1. *Differences between dairy and cutaneous propionibacteria*

Characteristic	Dairy propionibacteria	Cutaneous propionibacteria
Respiration	Microaerophilic or facultative	Anaerobic
Pathogenicity	-	+
Colony colour	Cream, yellow, orange, red, brown	Pinkish
DNA (G+C mol%)	65-68	58-64
Esculin hydrolysis	-[a]	+
Gelatin liquidation	+[b]	-[c]
Growth optimum (°C)	30-32	36-37

a) *Propionibacterium avidum* is positive. b) *Propionibacterium granulosum* and *Propionibacterium lymphophilum* are negative. c) *Propionibacterium cyclohexanicum* is positive.

4.1 Characteristics

Propionibacteria are Gram-positive, irregular, non-spore-forming, non-motile, catalase-positive rods (Vorobjeva, 1999). Under the microscope propionibacteria are often seen in characteristic arrangements; V or Y shaped or like Chinese characters. Depending on growth stage and on environment, their morphology and size can vary distinctly (Jan *et al.*, 2007). The main metabolites are propionic acid, acetic acid and carbon dioxide, but succinic acid, formic acid and vitamin B_{12} are also formed (Vorobjeva, 1999). The dairy propionibacteria include the following species:

- *Propionibacterium freudenreichii*
- *Propionibacterium jensenii*
- *Propionibacterium thoenii*
- *Propionibacterium acidipropionici*
- *Propionibacterium cyclohexanicum*

Distinguishing between these species is not always an easy task, but some species-specific characteristics can be identified:

P. freudenreichii
The most wide-spread species, used as starter culture in Swiss type cheese; the highest thermal resistance; non-haemolytic; colonies usually white or beige; subspecies *freudenreichii* and *shermanii*, can be separated by lactose fermentation (*freudenreichii* -, *shermanii* +).

P. jensenii
Rather diverse, strains originally described as separate species; both aerobic and anaerobic; ß-haemolytic; colonies creamy to yellow; some strains produce extracellular slime.

P. thoenii
ß-haemolytic; slowest growing species; colonies yellow, orange, red or brown.

P. acidipropionici
Weak or negative catalase reaction; grows aerobically; colonies beige to orange; some strains produce extracellular slime.

P. cyclohexanicum
Negative catalase reaction; produce lactic acid; very acid-tolerant; colonies white or creamy.

Figure 2 shows three strains of each of the first four dairy propionibacteria species on SL agar.

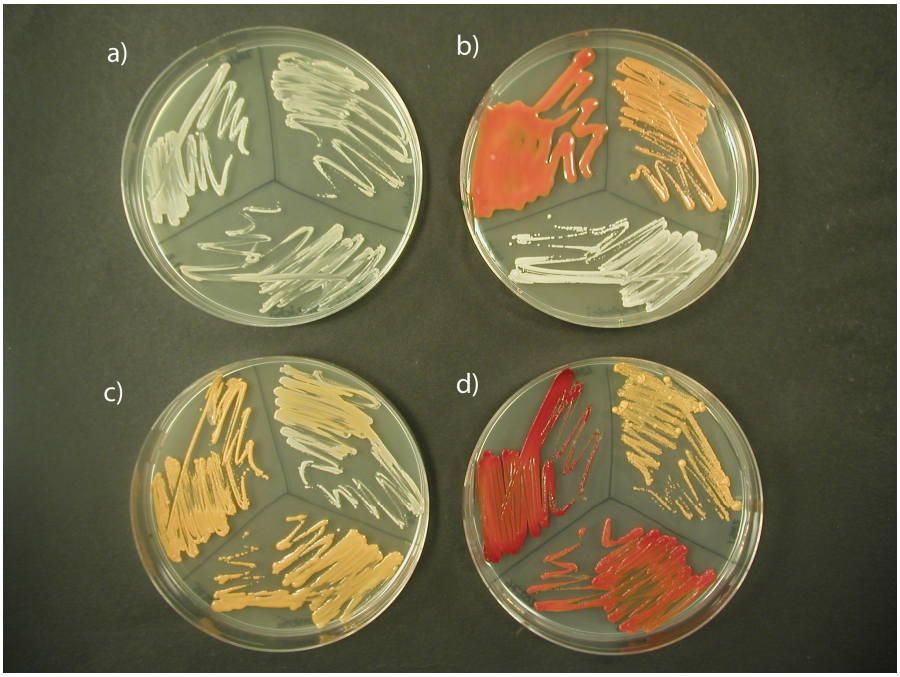

Figure 2. Three strains from each dairy propionibacteria species on each agar plate. a) *P. freudenreichii,* b) *P. acidipropionici,* c) *P. jensenii,* d) *P. thoenii.*

4.1.1 Growth Conditions

Dairy propionibacteria can utilise many different carbon sources, for example glucose, fructose and glycerol, but the preferred substrate is lactate (Jan *et al.*, 2007). A suitable medium contains 1% sodium lactate, 1% tryptone, 0.5% yeast extract and 0.5% KH_2PO_4. This will support good growth, but depending on the purpose of the cultivation, other media can be more suitable. Evaluation of five type strains of dairy propionibacteria on three different substrates showed differences in both antifungal activity and metabolite production (**I**). Sodium lactate medium (SL), as described above, was compared with commercial de Man Rogosa Sharp (MRS) and a modified version of MRS where the acetate was removed (MRS-ac). The carbon source of SL is lactate and in both MRS versions it is glucose. Growth was observed with all three media, but the production of acids differed depending on carbon source. *P. thoenii* showed weak growth, but apart from that, all strains produced the highest amounts of propionic acid and acetic acid in the SL medium. The strains were also inoculated on agar plates with the same

media and after growth they were overlaid with soft agar containing mould spores or yeast cells to measure the inhibitory capacity. In this assay, inhibition by propionibacteria grown on SL was least effective. Only the spoilage mould *Aspergillus fumigatus* was affected by some propionibacteria on SL. Fungal inhibition on MRS and MRS-ac was visible for all indicator fungi, but varied in strength and no superior medium could be selected (Fig. 3). The initial pH of all three media was around 6. After growth of propionibacteria the pH was strongly reduced on plates with MRS and MRS-ac, but not at all on SL, probably due to higher buffering capacity of the SL medium. At pH 6, only 6.9% of propionic acid and 5.4% of acetic acid is present in the undissociated form, *i.e.* that with antimicrobial effect.

The pH value itself is also a factor influencing growth of propionibacteria. The optimal range for growth reaches from 8.5 down to 5.1 (Jan *et al.*, 2007). Depending on the desired end product, optimal conditions can vary greatly. To achieve maximum yield of exopolysaccharides from *P. acidipropionici*, the optimal pH has to be narrowed to 5.3–6.5 (Gorret *et al.*, 2001).

The temperature optimum for dairy propionibacteria is normally around 30°C, but growth is possible between 15°C and 40°C, depending on strain and other growth conditions (Jan *et al.*, 2007). A study with 10 isolates of *P. freudenreichii* subsp. *shermanii* showed that clearly different thermotolerance could be identified for different strains within the same subspecies (Anastasiou *et al.*, 2006).

The most acid- and temperature-tolerant dairy species, *P. cyclohexanicum*, can cause spoilage of otherwise fairly stable food products, such as fruit juices. Pre-treatment at moderately high temperature resulted in higher cell survival after treatment at 85°C, 90°C or 95°C. However, in orange juice, the cells which were not pre-treated survived to the same extent as the pre-treated cells (Walker & Phillips, 2007).

The production of propionic acid and vitamin B_{12} requires different conditions. Maximum yield of propionic acid requires a completely anaerobic environment at 37°C, while optional conditions for vitamin B_{12} production are aerobic conditions at 40°C (Quesada-Chanto *et al.*, 1994).

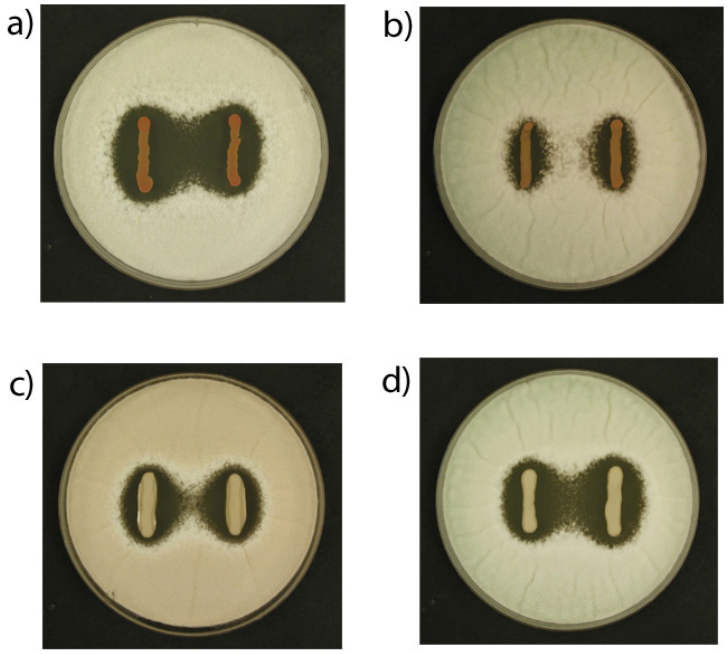

Figure 3. Type strains of dairy propionibacteria on different media. *P. thoenii* 20276 against *A. fumigatus* on (a) MRS and (b) MRS without acetate, and *P. jensenii 20535* against *A. fumigatus* on (c) MRS and (d) MRS without acetate.

As previously reported (**I**) different carbon sources will result in different propionic acid concentrations. A comparison between lactate, glycerol and sugarcane molasses showed that lactate resulted in the highest final concentration of propionic acid (Coral *et al.*, 2008). However when the aim was to produce pure propionic acid, without acetic acid as a by-product, glycerol was the preferred substrate. Furthermore, growth on sugarcane molasses gave the highest biomass yield (Coral *et al.*, 2008).

Atmosphere composition also affects metabolite production. Three strains of *P. freudenreichii* subsp. *shermanii* were grown in air and compared with growth under N_2 pressure. Production of acetic acid and carbon dioxide was higher, but production of propionic acid was lower during growth in air compared with N_2 atmosphere (Benjelloun *et al.*, 2007).

The ability to use dairy propionibacteria in probiotic products requires tolerance to bile salts. Leverrier *et al* (2003) showed that a pretreatment with moderate concentrations of bile salts or with thermal or detergent exposure greatly increased survival in otherwise lethal concentrations of bile salts. In

contrast, acid pretreatment seemed to sensitise the cells to the effects of bile salts.

4.1.2 Metabolism

During growth of propionibacteria on lactate the molar ratio of metabolites is:

$$3 \text{ lactate} \longrightarrow 2 \text{ propioniate} + 1 \text{ acetate} + 1\ CO_2 + 1\ H_2O$$

(Piveteau, 1999)

In reality, precisely this outcome is rare, since many factors affect the relative concentrations. If another carbon source is present the balance will be different. Growth on sugars such as glucose, lactose or galactose gives a much higher energy yield than lactate and results in higher growth rate and biomass. However, the relative amount of propionic and acetic acid is larger in lactate fermentation (Piveteau, 1999). If both lactate and sugars are available in the same substrate, lactate is the preferred substrate for propionibacteria (Piveteau, 1999).

Propionic acid fermentation is not exclusively found in the genus *Propionibacterium*, but in many other genera such as *Clostridium, Bacillus, Pseudomonas* and *Bacteroides* (Kim *et al.*, 2010; Salazar *et al.*, 2009).

Dairy propionibacteria are aerotolerant to varying extents. It has now been established that some propionibacteria can be adapted to growth under aerobic conditions (Vorobjeva, 1999). The details of this pathway are not fully understood, but a shift from anaerobic to aerobic cultivation gave a distinct metabolic switch. The propionic acid was completely decomposed and the production of acetic acid was enhanced and accompanied by formation of malic acid (Ye *et al.*, 1999).

Growth on glycerol also results in acid yield different from that found during lactate fermentation. Comparative studies have found glycerol to be the superior substrate for propionic acid production (Himmi *et al.*, 2000; Barbirato *et al.*, 1997). By using ^{13}C-labelled glycerol the homopropionic fermentation from glycerol was confirmed (**III**). Nuclear magnetic resonance (NMR) analysis after growth on glycerol (50% 1, 3-labelled and 50% 2-labelled) detected only labelled propionic acid, no other compounds could be found.

Beside propionic acid, vitamin B_{12} is probably the most important metabolite of propionibacteria. It is produced in large amounts compared with the level in most other microorganisms, and is essential for both the

propionibacteria themselves and for humans. A study of the synthesis in dairy propionibacteria reveals a short lag phase in parallel with growth. In the beginning, growth is dependent on external vitamin sources. Vitamin B_{12} synthesis is only activated when enough energy is produced (Vorobjeva, 1999). The production of vitamin B_{12} is vital for the key reaction of propionic acid fermentation in propionibacteria; the isomerisation of succinyl-CoA to methyl malonyl-CoA (Vorobjeva, 1999). In addition, propionibacteria have been found to produce vitamin B_9, commonly known as folic acid (Hugenholtz *et al.*, 2002). Although the significance of folic acid for propionibacteria is still poorly known, its usefulness for humans is obvious. Folic acid, like vitamin B_{12}, is necessary for haematopoesis (production of blood cells) and pregnant women are recommended an increased intake of folic acid to prevent neural tube defects in the foetus.

Dairy propionibacteria are also acknowledged producers of bacteriocins and other antimicrobial compounds during fermentation. Some examples of bacteriocins are jenseniin G (Ekinci & Barefoot, 2006), propionicin PLG-1 (Lyon *et al.*, 1993), propionicin T1 (Faye *et al.*, 2000) and thoeniicin 447 (Van der Merwe *et al.*, 2004), all produced by strains of *P. thoenii*, propionicin F (Brede *et al.*, 2004) and some unnamed bacteriocins from *P. freudenreichii* (Gwiazdowska & Trojanowska, 2006).

The production of 3-phenyl lactic acid with antifungal potential from dairy propionibacteria was demonstrated in the present work (**II**). In addition, two antifungal cyclic dipeptides and seven antifungal linear peptides were discovered. Further investigation confirmed the peptides to be derived from the growth medium (SL). One component of this medium is tryptone, which contains a pancreatic digest of ß-casein. Three of the linear peptides identified had sequences found in casein, so they probably originated from this source. Determination of the concentration of the cyclic dipeptide cyclo(Phe-Pro) showed similar amounts in SL media before and after growth of *P. jensenii* (14.7 µg/mL and 15.7 µg/mL respectively) and it was therefore assumed to also originate from the medium (**II**).

4.1.3 Related Species

Dairy propionibacteria share some features with bacteria from other genera. The phylogenetic relationship with *Corynebacterium* was mentioned previously. Some distinct characteristics easily separate them from propionibacteria: aerotolerance, G+C content in their DNA and colony colour (Vorobjeva, 1999).

The genus closest to dairy propionibacteria, both with regard to physiological similarities and their existence in similar environments, is

Lactobacillus. Dairy propionibacteria and lactobacilli are found in dairy products, but also in nature on grass or soil. They both grow well anaerobically on similar substrates and form very similar colonies, unless the propionibacteria are from one of the coloured species. Even so, since lactobacilli grow much faster they usually outcompete propionibacteria.

On the same substrate, the only reliable way to separate the different bacteria is probably by molecular techniques. A polymerase chain reaction (PCR) amplification of separate colonies, followed by nucleotide sequencing, would give a reliable answer (Rossi *et al.*, 1999). Separation of these species from the same culture is possible with sufficiently selective substrates. This thesis shows that strains of *P. jensenii* and *Lactobacillus fermentum* can be separated on selective substrates after co-culturing in mini silos for moist grain preservation (**IV**). The use of Rogosa medium for enumeration of *L. fermentum* enabled selective growth, as *P. jensenii* was not able to grow on this medium, probably due to the high acetate concentration and low pH. Finding a selective substrate for propionibacteria was more difficult. The solution in this work was to create antibiotic resistance by repeated selections on rifampicin- and streptomycin-containing agar. The use of SL agar supplemented with rifampicin and streptomycin made it possible to enumerate even *P. jensenii* on a selective medium. Figure 4 shows growth on SL agar without antibiotics after prolonged incubation.

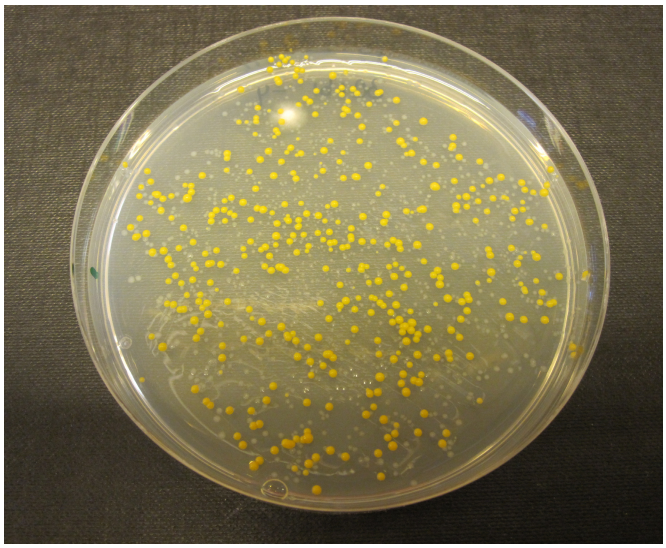

Figure 4. Growth of *L. fermentum* T14 (white colonies) and *P. jensenii* MP28RS02 (yellow colonies) on SL agar after prolonged incubation (two weeks).

4.2 Applications

Before using microorganisms in any food- or feed-related application, a number of questions need to be answered:

1. Is the microorganism safe to man and environment?
2. Can the microorganism be cultured and stored with maintained viability and desired characteristics?
3. Can the experiment be performed at full scale without affecting the results?

For the first question, thorough investigation will be needed. As early as possible in the development process, the selected microorganisms need to be evaluated regarding toxin production, exposure hazard to humans, animals and the environment and resistance to therapeutic antibiotics (Melin *et al.*, 2007). Before continued investigation, is also necessary to evaluate the economic viability of the end-product (Gautam & Sharma, 2009). In the European Union (EU), the European Food Safety Authority (EFSA) has introduced a list of safety assessed microorganisms for use in food or feed, called Qualified Presumption of Safety (QPS) (European Food Safety Authority, 2007). Before being listed in QPS the microorganism has undergone rigorous examination regarding the above-mentioned features and requires no further evaluation by the EFSA if chosen for an application (European Food Safety Authority, 2007). So far, two of the dairy propionibacteria, *P. freudenreichii* and *P. acidipropionici*, have been granted QPS status. For the use of microorganisms in feed very detailed legislation has already been adopted in the EU (Wessels *et al.*, 2004). Regulations on the use of microorganisms in food are still a concern for each individual EU country, with large variations ranging from strict official demands to no legislation covering starter cultures at all (Wessels *et al.*, 2004). In any case, until the other dairy propionibacteria species receive QPS status their application can still be investigated, based on their close relationship to *P. freudenreichii* and *P. acidipropionici*, which will probably make their inclusion in QPS somewhat faster. Melin *et al.* (2007) recommend a search for putative biopreservation organisms in groups where approved species already exist.

The second question in the list above concerns the culture and storage properties of the microorganism. The mode of action, as well as the influence on both intended and unintended targets, should be evaluated. For many microorganisms intended for biopreservation, the strongest effect is due to the production of weak organic acids. The activity is highly dependent on concentration, pH and target organism. The three most important organic acids in biopreservation (lactic, acetic and propionic acid)

and their effects on eight different spoilage fungi were investigated at different pH and different concentrations in the present work (**I**). The major finding was the unexpected result that *Penicillium roqueforti* was the most sensitive species tested. This is a fungus that is usually regarded as being very pH-tolerant and resistant to acids (Pitt & Hocking, 2009). Not surprisingly, there was a large difference in the inhibitory effect of the organic acids between different pH values. At pH 3, an acid concentration of 20 mM or lower was usually enough for total inhibition, whereas at pH 7, 500 mM acid were seldom sufficient for visible inhibition. Among the acids tested, propionic acid was clearly the most potent. Due to its high pK_a, value, propionic acid has a higher proportion of undissociated molecules at higher pH. Evaluation of the acid production and antifungal activity of five type strains of dairy propionibacteria in an overlay assay revealed that *P. acidipropionici*, *P. thoenii* and *P. jensenii* were the strongest fungal inhibitors. Since *P. jensenii* was the best propionic acid producer among those, strains of that species should be possible candidates for biopreservation applications.

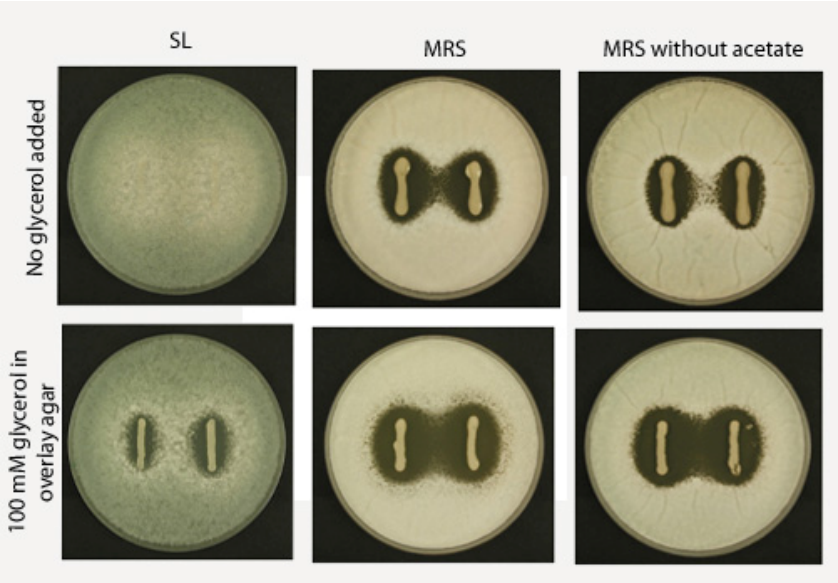

Figure 5. Inhibition of *A. fumigatus* by *P. jensenii* on different substrates with or without addition of glycerol.

The influence of glycerol on antifungal activity was evaluated in a subsequent study (**III**). Glycerol is a non-toxic, almost tasteless, odourless substance, already used as a food additive and easy to obtain in large

quantities due to its occurrence as by-product in biodiesel production, a growing industry (Behr *et al.*, 2008). In addition, a few lactobacilli use glycerol in the production of 3-hydroxypropionaldehyde, also known as reuterin, a potent antimicrobial substance (Vollenweider & Lacroix, 2003). The conversion of glycerol by glycerol dehydratase is coenzyme B_{12}-dependent and the genetic organisation of the dehydratase genes is similar in all organisms (Daniel *et al.*, 1999). The similarity between dairy propionibacteria and lactobacilli led to a closer investigation of a combination of dairy propionibacteria and glycerol. An initial experiment with addition of glycerol to the overlay agar in assays with SL, MRS and MRS-ac showed distinctly improved antifungal activity (Fig. 5, unpublished results). In the following study only SL was used (**III**). Since previous results showed antifungal activity without glycerol on the other media, SL would be the most appropriate medium on which to study the glycerol-effect. Addition of glycerol to the antifungal assays strongly enhanced the effect in a dose-dependent manner. The addition of only 50 mM glycerol in the overlay agar gave a visible effect on fungal inhibition for some propionibacteria and some indicator fungi. The most sensitive of the indicator fungi was the mould *Penicillium commune*, while the yeast *Rhodotorula mucilaginosa* showed a weak response even at a glycerol concentration of 500 mM. An attempt to find gene sequences equivalent to those responsible for reuterin production in lactobacilli, by chromosomal DNA hybridisation, was unsuccessful. In an NMR analysis with ^{13}C-labelled glycerol no compound other than propionic acid was found. Thus, it is most probably the homofermentative production of propionic acid, which rapidly lowers the pH and leads to strong organic acid activity, that is the mechanism behind the enhanced fungal inhibition effect seen after addition of glycerol.

Figure 6. A mini silo containing moist wheat grain with a biopreservation culture and glycerol.

Before further investigations are performed, it would also be a good idea to ensure the microorganism can be formulated in an applicable way and to investigate the survival after storage (Schoug *et al.*, 2008). When applying a microorganism to food or feed it is also important to make sure that no negative effects appear on the product. The organoleptic properties and the shelf-life should be intact or even improved.

A strain of *P. jensenii* was chosen for a small-scale experiment in combination with *L. fermentum* as a

biopreserving culture in mini silos of moist grain (Fig. 6) (**IV**). The addition of glycerol was also evaluated. Both bacteria grew faster and to higher densities at higher moisture content (approximately 30%). This resulted in higher amounts of organic acids produced, and consequently lower pH. The addition of glycerol accelerated the acid production in the beginning of the experiment but after six weeks the acid concentration was equal to that in mini silos without glycerol. An unexpected result was that production of propionic acid in mini silos with low moisture content (approximately 25%) was initiated after only four weeks. In corresponding mini silos without glycerol no propionic acid could be detected even after six weeks of incubation. Even if no significant effect on the growth of the added target organism, *P. roqueforti*, could be detected, continued investigations of the system with other bacterial combinations and other glycerol concentrations are justifiable.

Before registration and application in the intended industry, a final step is required. The system need to be tested at full scale and also with all expected environmental factors present, preferably on the site of future use.

4.2.1 Applications in Food

Dairy propionibacteria are naturally found in dairy environments, so their application in an environment such as cheese should not meet too many obstacles. An attempt to prevent spoilage by yeast in Kareish cheese with a mixed culture of *P. thoenii* and two different lactobacilli gave promising results. The combinations prolonged shelf-life and the cheese could be stored for 30 days without yeast spoilage. Moreover, characteristics such as flavour and texture were improved (El-Shafei *et al.*, 2008).

Another study used the metabolites from a culture of *P. thoenii* as the preserving additive. Use in the soft cheese Domiati revealed the same positive results: prolonged shelf-life and enhanced organoleptic properties (Tawfik *et al.*, 2004). A combination of *P. jensenii* and *Lactobacillus paracasei* subspecies *paracasei* had an inhibitory effect on yeast spoilage on the surface of cheese and in yoghurt. No increase in yeast cells could be detected in any of the systems, and the texture of the yoghurt was improved (Miescher Schwenninger & Meile, 2004).

The use of *P. jensenii* and *P. thoenii* as the only microorganisms in a starter culture for yoghurt was less successful due to the slow growth of the propionibacteria. However, in combination with a commercial starter culture for yoghurt some positive effects of the propionibacteria could be seen. No negative influence on either physical or chemical properties of the

products was detected, while the number of bacteria from the starter culture was kept intact (Ekinci & Gurel, 2008).

In sourdough bread, a combination of *P. freudenreichii* subspecies *shermanii* and *Lactobacillus rhamnosus* was effective in the inhibition of yeast and *Bacillus* species (Soumalainen & Mäyrä-Mäkinen, 1999).

The addition of dairy propionibacteria to the LAB starter culture for fermentation of vegetables demonstrated a wide range of positive effects such as inhibition of unwanted microorganisms, increased content of vitamin B_{12} and folic acid and in some cases also improved organoleptic properties (Babuchowski *et al.*, 1999).

Evaluations of the satiety effects of dairy beverages fermented with *P. freudenreichii* and *Lactobacillus acidophilus* revealed that the test subjects showed significantly increased satiety effect after consumption of the product compared with a placebo (Ruijschop *et al.*, 2008).

4.2.2 Applications in Feed

The use of dairy propionibacteria in feed, both as a biopreserving culture and directly fed, has also been evaluated.

A co-culture of *P. acidipropionici* and *Lactobacillus plantarum* was applied to different grain silages to evaluate the aerobic stability. The *P. acidipropionici* was also used separately and the results showed that the pure propionibacteria culture was effective and improved aerobic stability of wheat, sorghum and maize silages. In combination with *L. plantarum* no effect on aerobic stability was found (Filya *et al.*, 2004).

A commercial starter culture for maize silage containing *P. acidipropionici* and *L. plantarum* was evaluated at different inoculation levels. Inoculation with 1.5×10^{10} colony forming units (cfu)/g, half the recommended level, resulted in the most effective fermentation, since it gave the highest acid concentrations. Another positive effect was the decreased cost following lower inoculation levels (Rowghani *et al.*, 2008).

Another application for dairy propionibacteria is the use of pure cultures as a nutritional feed complement. Direct feeding of a *P. jensenii* culture to calves had a positive impact on calf live-weight gain (Adams *et al.*, 2008).

Direct feeding of a propionibacteria culture (P169) to early lactation dairy cows showed no effect on milk production, but an increase in feed efficiency, with test cows consuming less feed for the same level of milk yield (Weiss *et al.*, 2008). The same propionibacterium (P169) increased milk yield and caused complex hormone and metabolic changes, with the results depending on dose and individual cow (Aleman *et al.*, 2007).

5 Conclusions

The antifungal properties of dairy propionibacteria can increase their applicability as biopreservation agents. This thesis has investigated different aspects of their antifungal activity. Weak, organic acids are responsible for most of the inhibition of fungi. This effect is highly dependent on pH. In a low pH a greater proportion of the acid is present as undissociated molecules, hence, the inhibitory activity of a weak acid increases with decreasing pH. Propionic acid is the most potent inhibitor.

Penicillium roqueforti was the fungus most sensitive to lactic, acetic and propionic acid (**I**). The antifungal activity of five dairy propionibacteria was dependent on substrate and showed species variation: *Propionibacterium thoenii* was most effective in fungal inhibition, but *Propionibacterium jensenii* produced the most acids (**I**).

In addition to propionic acid, dairy propionibacteria also produce other antifungal compounds. Active compounds from dairy propionibacteria were identified by separation on a solid phase extraction column and subsequent HPLC fractionation. (**II**). *P. jensenii* produced the antifungal compound 3-phenyl lactic acid. Several antifungal peptides were also identified in the growth medium (SL).

Addition of glycerol strongly enhanced the antifungal activity of dairy propionibacteria in a dose-dependent manner (**III**). No compound other than propionic acid could be detected after growth on glycerol by dairy propionibacteria. However, growth on glycerol switched the metabolism to a homopropionic production, with no acetic acid detected in the spent medium.

Dairy propionibacteria and lactobacilli could potentially be a useful combination in the biopreservation of moist grain. Growth of *P. jensenii* MP28RS02 and *Lactobacillus fermentum* T14 in moist wheat grain (MC 32%) led to formation of lactic, acetic and propionic acid and decreased pH from

6.2 to 4.1 after six weeks (**IV**). The addition of 1% glycerol enhanced the potential preserving capacity with an even lower pH (4.0) and an earlier formation of acids.

5.1 Future perspectives

The growing population in the world presents a major challenge. To meet the need for increased food production we have to concentrate our efforts at the beginning of the production chain. A more effective industry that is also committed to achieving a sustainable environment will be required. Agricultural research aimed at maximising the utilisation of resources, crops and methods giving the highest yields and development of effective ways of storing, processing and distributing the products are essential for the future. We also need to focus on effective solutions for stable storage of food and feed, leading to a higher utilisation ratio and less waste and meeting the demands of consumers who are getting more interested and more informed about additives in food.

Demands for biological preservatives can be met by the use of microorganisms, presenting a healthy alternative to chemicals by the production of storage-stabilising compounds and substances enhancing the nutritional value.

Dairy propionibacteria are organisms with a great future ahead of them. They possess a number of positive properties, and there is probably an even wider range of advantageous features waiting to be discovered. Experimental use of dairy propionibacteria as biopreservative cultures is giving promising results.

References

Adams, M.C., Luo, J., Rayward, D., King, S., Gibson, R. & Moghaddam, G.H. (2008). Selection of a novel direct-fed microbial to enhance weight gain in intensively reared calves. *Animal Feed Science and Technology* 145, 41-52.

Adams, M.R. & Moss, M.O. (2000). *Food Microbiology*. Second edition. Cambridge, UK: The Royal Society of Chemistry.

Aleman, M.M., Stein, D.R., Allen, D.T., Perry, E., Lehloenya, K.V., Rehberger, T.G., Mertz, K.J., Jones, D.A. & Spicer, L.J. (2007). Effects of feeding two levels of propionibacteria to dairy cows on plasma hormones and metabolites. *Journal of dairy research* 74(2), 146-153.

Anastasiou, R., Leverrier, P., Krestas, I., Rouault, A., Kalantzopoulos, G., Boyaval, P., Tsakalidou, E. & Jan, G. (2006). Changes in protein synthesis during thermal adaption of *Propionibacterium freudenreichii* subsp. *shermanii*. *International Journal of Food Microbiology* 108(3), 301-314.

Andersson, R., Andrén, O., Bergström, L., Kirchmann, H., Kätterer, T., Arvidsson, T., Kyllmar, K., Torstensson, G. & Stenström, J. (2009). Argumentationen för ekoodling är osaklig. *Svenska Dagbladet*. Stockholm

Babuchowski, A., Laniewska-Moroz, L. & Warminska-Radyko, I. (1999). Propionibacteria in fermented vegetables. *Lait* 79, 113-124.

Barbirato, F., Chedaille, D. & Bories, A. (1997). Propionic acid fermentation from glycerol: comparison with conventional substrates. *Applied Microbiology and Biotechnology* 47(4), 441-446.

Behr, A., Eilting, J., Irawadi, K., Leschinski, J. & Lindner, F. (2008). Improved utilisation of renewable resources: New important derivates of glycerol. *Green Chemistry* 10(1), 13-30.

Bengtsson, M. & Persson, D. (2009). *Växtskyddsmedel i jordbruket 2008*. Statistiska centralbyrån. (Statistiska meddelanden)

Benjelloun, H., Rabe Ravelona, M. & J-M., L. (2007). Characterization of growth and metabolism of commercial strains of propionic acid bacteria by pressure measurement. *Engineering in Life Science* 7(2), 143-148.

Brede, D.A., Faye, T., Johnsborg, O., Ödegård, I., Nes, I.F. & Holo, H. (2004). Molecular and genetic characterization of propionicin F, a bacteriocin from *Propionibacterium freudenreichii*. *Applied and Environmental Microbiology* 70(12), 7303-7310.

Brown, K. (2004). *Penicillin man - Alexander Fleming and the antibiotic revolution*. Gloucestershire: Sutton Publishing Limited.

Buckel, W. (1999). Anaerobic energy metabolism. In: Lengeler, J.W.*, et al.* (Eds.) *Biology of the prokaryotes*. Stuttgart: Blackwell science Ltd.

Coral, J., Karp, S.G., Porto de Souza Vandenberghe, L., Parada, J.L., Pandey, A. & Soccol, C.R. (2008). Batch fermentation model in propionic acid production by *Propionibacterium acidipropionici* in different carbon sources. *Applied Biochemistry and Biotechnology* 151(2), 333-341.

Daniel, R., Bobik, T.A. & Gottschalk, G. (1999). Biochemistry of coenzyme B_{12}-dependent glycerol and diol dehydratases and organization of the encoding genes. *FEMS Microbiology Reviews* 22, 553-566.

Danielsson, D. & Kjellander, J. (1993). *Klinisk mikrobiologi*. Fifth edition. Falköping: Almqvist and Wiksell Medicine.

Davidson, P.M. (2001). Chemical preservatives and natural antimicrobial compounds. In: Doyle, M.P.*, et al.* (Eds.) *Food Microbiology: Fundamentals and Frontiers*. Washington D.C.: ASM Press. pp. 593-627.

Demain, A.L. & Adrio, J.L. (2008). Contributions of microorganisms to industrial biology. *Molecular Biotechnology* 38(1), 41-55.

Denny, A., Aisbitt, B. & Lunn, J. (2008). Mycoprotein and health. *British Nutrition Foundation - Nutrition bulletin* 33, 298-310.

Einarsson, P. (1996). Från soldrift till olja- och tillbaka igen? In: Lundgren, L. (Ed.) *Maten och miljön: Råvaror, resurser, recept*. Stockholm: Naturskyddsföreningen.87.

Ekinci, F.Y. & Barefoot, S.F. (2006). Fed-batch enhancement of jenseniin G, a bacteriocin produced by *Propionibacterium thoenii* (*jensenii*) P126. *Food Microbiology* 23, 325-330.

Ekinci, F.Y. & Gurel, M. (2008). Effect of using propionic acid bacteria as an adjunct culture in yogurt production. *Journal of Dairy Science* 91, 892-899.

El-Shafei, K., Abd El-Gawad, M.A.M., Dabiza, N., Sharaf, O.M. & Effat, B.A. (2008). A mixed culture of *Propionibacterium thoenii* P-127, *Lactobacillus rhamnosus* and *Lactobacillus plantarum* as protective cultures in kareish cheese. *Polish Journal of Food and Nutrition Sciences* 58(4), 433-441.

European Food Safety Authority (2007). Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. *The EFSA Journal* 587, 1-16.

Faye, T., Langsrud, T., Nes, I.F. & Holo, H. (2000). Biochemical and Genetic Characterization of Propionicin T1, a New Bacteriocin from *Propionibacterium thoenii*. *Applied and Environmental Microbiology* 66(10), 4230-4236.

Feng, X.M., Eriksson, A.R.B. & Johan, S. (2005). Growth of lactic acid bacteria and *Rhizopus oligosporus* during barley tempeh fermentation. *International Journal of Food Microbiology* 104, 249-256.

Filya, I., Sucu, E. & Karabulut, A. (2004). The effect of *Propionibacterium acidipropionici*, with or without *Lactobacillus plantarum*, on the fermentation and aerobic stability of wheat, sorghum and maize silages. *Journal of applied microbiology* 97, 818-826.

Freese, E., Sheu, C.W. & Galliers, E. (1973). Function of lipophilic acids as antimicrobial food additives. *Nature* 241(2), 321-325.

Gautam, N. & Sharma, N. (2009). Bacteriocin: safest approach to preserve food products. *Indian Journal of Microbiology* 49, 204-211.

Ghalfi, H., Benkerroum, N., Ongena, M., Bensaid, M. & Thonart, P. (2010). Production of three anti-listerial peptides by *Lactobacillus curvatus* in MRS broth. *Food Research Journal* 43, 33-39.

Glazer, A.N. & Nikaido, H. (2007). *Microbial biotechnology, Fundamentals of applied microbiology*. Second edition. New York: Cambridge University Press.

Gorret, N., Maubois, J.L., Engasser, J.M. & Ghoul, M. (2001). Study of the effects of temperature, pH and yeast extract on growth and exopolysaccharides production by *Propionibacterium acidi-propionici* on milk microfiltrate using a response surface methodology. *Journal of Applied Microbiology* 90, 788-796.

Gwiazdowska, D. & Trojanowska, K. (2006). Antimicrobial activity and stability of partially purified bacteriocins produced by *Propionibacterium freudenreichii* ssp. *freudenreichii* and ssp. *shermanii*. *Lait* 86, 141-154.

Hansen, E.B. (2002). Commercial bacterial starter cultures for fermented foods of the future. *International Journal of Food Microbiology* 78, 119-131.

Hata, T., Tanaka, R. & Ohomomo, S. (2010). Isolation and characterization of plantaricin ASM1: A new bacteriocin produced by *Lactobacillus plantarum* A-1. *International Journal of Food Microbiology* 137, 94-99.

Himmi, E.H., Bories, A., Boussaid, A. & Hassani, L. (2000). Propionic acid fermentation of glycerol and glucose by *Propionibacterium acidipropionici* and *Propionibacterium freudenreichii* ssp. *shermani*. *Applied Microbiology and Biotechnology* 53, 435-440.

Hugenholtz, J., Hunik, J., Santos, H. & Smid, E. (2002). Nutraceutical production by propionibacteria. *Lait* 82, 103-112.

Jan, G., Lan, A. & Leverrier, P. (2007). Dairy propionibacteria as probiotics. In: Saarela, M. (Ed.) *Functional dairy products*. Woodhead Publishing 2. pp. 165-194.

Jarvis, G.N., Strömpl, C., Moore, E.R.B. & Thiele, J.H. (1998). Isolation and characterisation of obligately anaerobic, lipolytic bacteria from the rumen of red deer. *Systematic and Applied Microbiology* 21, 135-143.

Jonsson, N. & Pettersson, H. (1999). *Utvärdering av olika konserveringsmetoder för spannmål: baserad på analyser av hygienisk kvalitet*. Uppsala: Jordbrukstekniska institutet. (JTI-rapport. Lantbruk & industri, 263)

Jonsson, U. (1994). *Från hästkärra till skördetröska: jordbruk och jordbruksarbete under 1900-talet*. Stockholm: Ordfront (Bryta, bygga, bo, svensk historia underifrån)

Kim, M.D., Song, M., Jo, M., Shin, S.G., Khim, J.H. & Hwang, S. (2010). Growth condition and bacterial community for maximum hydrolysis of suspended organic materials in anaerobic digestion of food waste-recycling wastewater. *Applied Microbiology and Biotechnology* 85(5), 1611-1618.

Kumari, A. & Prakash Garg, A. (2007). A bacteriocin from *lactococcus lactic* CCSUB94 isolated from milk and milk products. *Research Journal of Microbiology* 2(4), 375-380.

Käck-Brolund, I. (1991). *Sambandet mat och miljö har ett pris*. Stockholm: Konsumentgillesförbundet.

Leverrier, P., Dimova, D., Picheraeu, V., Auffray, Y., Boyaval, P. & Jan, G. (2003). Susceptibility and adaptive response to bile salts in *Propionibacterium freudenreichii*: Physiological and proteomic analysis. *Applied and Environmental Microbiology* 69(7), 3809-3818.

Lyon, W.J., Sethi, J.K. & Glatz, B.A. (1993). Inhibition of Psychotrophic Organisms by Propionicin PLG-1, a Bacteriocin Produced by *Propionibacterium thoenii*. *Journal of Dairy Science* 76, 1506-1513.

Magan, N. & Aldred, D. (2007). Why do fungi produce mycotoxins? In: Jan, D*., et al.* (Eds.) *Food Mycology, A Multifaceted Approach to Fungi and Food*. Taylor and Francis group.

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

Meile, L., Le Blay, G. & Thierry, A. (2008). Safety assessment of dairy microorganisms: *Propionibacterium* and *Bifidobacterium*. *International Journal of Food Microbiology* 126, 316-320.

Melin, P., Sundh, I., Håkansson, S. & Schnürer, J. (2007). Biological preservation of plant derived animal feed with antifungal microorganisms: safety and formulation aspects. *Biotechnology Letters* 29, 1147-1154.

Miescher Schwenninger, S. & Meile, L. (2004). A mixed culture of *Propionibacterium jensenii* and *Lactobacillus paracasei* subsp. *paracasei* inhibits food spoilage yeasts. *Systematic and Applied Microbiology* 27, 229-237.

Moll, G., Ubbink-kok, T., Hildeng-Hauge, H., Nissen-Meyer, J., Nes, I.F., Konings, W.N. & Driessen, A.J.M. (1996). Lactococcin G is a potassium ion-conducting, two-component bacteriocin. *Journal of Bacteriology* 178(3), 600-605.

Nout, M.J.R. (2007). The colonizing fungus as a food provider. In: Jan, D., *et al.* (Eds.) *Food Mycology, A Multifaceted Approach to Fungi and Food*. Taylor and Francis group.

Nout, M.J.R. & Kiers, J.L. (2005). Tempe fermentation, innovation and functionality: update into the third millennium. *Journal of Applied Microbiology* 98, 789-805.

Pitt, J.I. & Hocking, A.D. (2009). *Fungi and food spoilage*. Third edition. New York: Springer.

Piveteau, P. (1999). Metabolism of lactate and sugars by dairy propionibacteria: A review. *Lait* 79, 23-41.

Quesada-Chanto, A., S-Afschar & A. Wagner, F. (1994). Optimization of a *Propionibacterium acidipropionici* continuous culture utilizing sucrose. *Applied Microbiology and Biotechnology* 42, 16-21.

Rahm, F. (2010). *Kortperiodisk sysselsättningsstatistik 4:e kvartalet 2009*. Statistiska centralbyrån. (Statistiska meddelanden)

Rinta-Koski, M., Beasley, S., Montonen, L. & Mantere-Alhonen, S. (2001). Propionibacteria isolated from rumen used as possible probiotics together with bifidobacteria. *Milchwissenschaft* 56(1), 11-13.

Rockström, J., Steffen, W., Noone, K., Persson, Å., Chapin, F.S., Lambin, E.F., Lenton, T.M., Scheffer, M., Folke, C., Schnellnhuber, H.J., Nykvist, B., de Wit, C.A., Hugher, T., van der Leeuw, S., Rodhe, H., Sörlin, S., Snyder, P.K., Costanza, R., Svedin, U., Falkenmark, M., Karlberg, L., Corell, R.W., Fabry, V.J., Hansen, J., Walker, B., Liverman, D., Richardson, K., Crutzen, P. & Foley, J.A. (2009). A safe operating space for humanity. *Nature* 461(24), 472-475.

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

Rossi, F., Torriani, S. & Dellaglio, F. (1999). Genus- and Species-Specific PCR-Based Detection of Dairy Propionibacteria in Environmental Samples by Using Primers Targeted to the Genes Encoding 16S rRNA. *Applied and Environmental Microbiology* 65(9), 4241-4244.

Rowghani, E., Zamiri, M.J., Khorvash, M. & Abdollahipanah, A. (2008). The effects of *Lactobacillus plantarum* and *Propionibacterium acidipropionici* on corn silage fermentation, ruminal degradability and nutrient digestibility in sheep. *Iranian Journal of Veterinary Research* 9(4), 308-315.

Rudquist, G. & Lundgren, L. (1996). Naturskyddsföreningen, maten och miljön. In: Lundgren, L. (Ed.) *Maten och miljön: råvaror, resuser, recept* Stockholm: Naturskyddsföreningen.87.

Ruijschop, R.M.A.J., Boelrijk, A.E.M. & te Giffel, M.C. (2008). Satiety effects of a dairy beverage fermented with propionic acid bacteria. *International Dairy Journal* 18, 945-950.

Salazar, N., Ruas-Madiedo, P., Kolida, S., Collins, M., Rastall, R., Gibson, G. & de los Reyes-Gavilán, C.G. (2009). Exopolysaccharides produced by *Bifidobacterium longum* IPLA E44 and *Bifidobacterium anmalis* subsp. *lactis* IPLA R1 modify the composition and metabolic activity of human faecal microbiota in pH-controlled batch cultures. *International Journal of Food Microbiology* 135(3), 260-267.

Schoug, Å., Fischer, J., Heipieper, H.J., Schnürer, J. & Håkansson, S. (2008). Impact of fermentation pH and temperature on freeze-drying survival and membrane lipid composition of *Lactobacillus coryniformis* Si3. *Journal of Industrial Microbiology and Biotechnology* 35, 175-181.

SIWI (2008). *50 Percent of Food is Wasted Causing Water, Food and Hunger Crisis, Says SIWI, FAO and IWMI* [online]. Stockholm Stockholm international water institute Available from: http://www.siwi.org/sa/node.asp?node=343 [Accessed 2010-03-31].

Soumalainen, T.H. & Mäyrä-Mäkinen, A.M. (1999). Propionic acid bacteria as protective cultures in fermented milks and breads. *Lait* 79, 165-174.

Stackebrandt, E., Cummins, C.S. & Johnsson, J.L. (2006). Bacteria: Family *Propionibacteriaceae*, the genus *Propionibacterium*. In: Dworkin, M.*, et al.* (Eds.) *The Procaryotes*. Springer Science.Vol 3.

Stratford, M. (1999). Traditional preservatives- Organic acids. In: Robinson, R.K. (Ed.) *Encyclopedia of Food Microbiology*. London: Academic Press. pp. 1729-1737.

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 3-phenyllactic acid. *Applied and Environmental Microbiology* 68(9), 4322-4327.

Swe, P.M., Cook, G.M., Tagg, J.R. & Jack, R.W. (2009). Mode of action of dysgalacticin: a large heat-labile bacteriocin. *Journal of Antimicrobial Chemotherapy* 63, 679-686.

Tawfik, N.F., Sharaf, O.M., Effat, B.A. & Mahanna, N.S. (2004). Preserving Domiati cheese using metabolites of *Propionibacterium thoenii* P-127. *Polish Journal of Food and Nutrition Sciences* 13/54(3), 269-272.

Tiwari, S.K. & Srivastava, S. (2008). Characterization of a bacteriocin from *Lactobacillus plantarum* strain LR/14. *Food Biotechnology* 22, 247-261.

Todorov, S.D. (2009). Bacteriocins from *Lactobacillus plantarum* - production, genetic organization and mode of action. *Brazilian Journal of Microbiology* 40, 209-221.

Walker, M. & Phillips, C.A. (2007). The growth of *Propionibacterium cyclohexanicum* in fruit juices and its survival following elevated temperature treatments. *Food Microbiology* 24, 313-318.

Van der Merwe, I.R., Bauer, R., Britz, T.J. & Dicks, L.M.T. (2004). Characterization of thoeniicin 447, a bacteriocin isolated from *Propionibacterium thoenii* strain 447. *International Journal of Food Microbiology* 92, 153-160.

Weiss, W.P., Wyatt, D.J. & McKelvey, T.R. (2008). Effect of feeding propionibacteria on milk porduction by early lactation dairy cows. *Journal of Dairy Science* 91(2), 646-652.

Wessels, S., Axelsson, L., Bech Hansen, E., De Vuyst, L., Laulund, S., Lähteenmäki, L., Lindgren, S., Mollet, B., Salminen, S. & von Wright, A. (2004). The lactic acid bacteria, the food chain, and their regulation. *Trends in Food Science and Technology* 15(10), 498-505.

Vollenweider, S. & Lacroix, C. (2003). 3-Hydroxypropionaldehyde: applications and perspectives of biotechnological production. *Applied Microbilogy and Biotechnology*

Vorobjeva, L.I. (1999). *Propionibacteria*. Kluwer Academic Publishers.

Ye, K., Shijo, M., Miyano, K. & Shimizu, K. (1999). Metabolic pathway of *Propionibacterium* growing with oxygen: Enzymes, ^{13}C NMR analysis, and its application for vitamin B_{12} production with periodic fermentation. *Biotechnology Progress* 15, 201-207.

Younts-Dahl, S.M., Galyean, M.L., Loneragan, G.H., Elam, N.A. & Brashears, M.M. (2004). Dietary supplementation with *Lactobacillus*- and *Propionibacterium*-based direct-fed microbials and prevalence of *Escherichia coli* O157 in beef feedlot cattle and on hides at harvest. *Journal of Food Protection* 67(5), 889-893.

Acknowledgements

This work was financially supported by the Foundation for Environmental Research (MISTRA), the Carl Trygger Foundation, Arla Foods and the Thematic Research Programme *MicroDrivE* (Microbially Derived Energy).

During my time as a PhD student at the Department of Microbiology I have had the opportunity to meet so many people who have taught me many things about science and life. I would like to express my deepest gratitude to all past and present members of the Department (and that is quite a few during my extended PhD education…) for creating a pleasant environment to work and develop in. And I really mean ALL of you!

Some people who contributed in a special way deserve special mention:

Johan Schnürer – Mitt varmaste tack för din entusiasm och uppmuntran! Du har en fantastisk förmåga att lyfta fram det positiva och se det användbara i de torftigaste resultat. Utan dig hade jag aldrig kommit dit jag är idag. Tack också för att du upplät ditt skrivrum till mig under slutfasen, en förvånansvärt inspirerande miljö!

Hans Jonsson – Tack för att du alltid har funnits till hands och tagit dig tid! Du är suverän på att visa intresse och omtanke, oavsett om det gäller baggar på en agarplatta eller om man har ätit någon lunch.

Anders Broberg – Tack för din hjälp på kemins område och för att du aldrig säger nej även om du har fullt upp.

Karin Jacobsson – Du har varit som en handledare och mer för mig, du är fantastisk! Tack för allt från praktisk labhjälp, manusbearbetning, tomatplantor och personligt engagemang.

Ingvar Sundh – Tack för att du helt frivilligt har engagerat dig i min avhandling och för utmärkt korrekturläsning.

Tack till **Jörgen Sjögren** för ett trevligt samarbete med en alltid lika glad och positiv kompis!

Anders Eriksson, min rumskamrat under de första åren. Du är en fantastisk vän som man kan prata om allt med, men även kunnig i allt vetenskapligt jag behövt fråga om. Tack också för din tveklösa generositet som nattchaufför!

Anna Schnürer – Tack för ditt sällskap i det minimala skrivrummet de senaste åren. Alla telefonsamtal, fysiska besök och GC-rör till trots, jag har trivts jättebra och uppskattat våra små pratstunder om Försäkringskassan och barnfilmer.

My Leffler – Ett varmt tack för att du tog mig med på ett oöverträffat äventyr i fåbodarnas rike och lät mig uppleva möten jag inte trodde var möjliga!

Den proffsiga tekniska hjälp jag har fått av **Anki, Barbro, Susanne, Sture** och **Ingemar** är värd ett riktigt stort tack: TACK!

Mina närmaste kollegor i LiFo, vad hade min tid på Mikro varit utan er? Tack **Katrin, Jesper, Torun** och **Stefan** som välkomnade mig i gruppen på ett strålande sätt, vem kan glömma konferensen i Cork? **Klara** och **Cecilia**, tack för att ni gjorde det så roligt att vara på labbet! Och tack till alla andra som befolkat gruppen under åren och som gjort att det alltid har varit kul att gå till jobbet: **Inger, Ulrika, Lisa, Jonas F, Jonas H, Petter, Volkmar, Xinmei, Jennifer, Matilda, Johanna, Åsa Sv, Johan D, Mia T, Henrik T, Henrik L, Greta, Bengt, Lars, Olga, Su-Lin, Therese, Tomas, Ivgeniia, Lotta J** och alla **exjobbare** och **sommarjobbare** (särskilt **Lisa H** och **Sandra**).

Ett stort tack till mina trivselkollegor **Karin N** och **Åsa K**, visst gjorde vi ett fantastiskt trivsamt Mikro tillsammans?

För att ni alltid har visat så mycket omtanke och förståelse, mitt varmaste tack till **Lotta L** och **Anna-Ida**!

Utöver alla fantastiska mikrober finns det några till som förtjänar mitt tack:

Mina härliga kursare och ordensvänner **Linda, Karin** och **Caroline**, tack för alla galna fester och roliga påhitt, länge leve Glöggkopparna!

Kristina, min allra käraste syster och bästa vän, tack för att man kan prata om allt med dig och att du förstår och bryr dig!

Tack min älskade familj **Uffe, Moa, Agnes** och **lillbuset**, vem du nu är, för att ni är så besvärliga och så underbara! Det är för er skull jag orkar kämpa på även om allt känns tungt. Moa och Agnes, nu vet jag verkligen vad som är meningen med livet!