SLU

Towards the development of a starter culture for improvement of grain storage through biopreservation

Malin Larsson

Institutionen för mikrobiologi
Sveriges lantbruksuniversitet

Uppsala

Examensarbete 2009:1

ISSN 1101-8151
ISRN SLU-MIKRO-EX–09/1–SE

SLU

Towards the development of a starter culture for improvement of grain storage through biopreservation

Malin Larsson

Thesis for Master of Science degree in Engineering Biology at Linköping Institute of Technology, Linköping University
Performed at the Department of Microbiology, SLU

Supervisor: Karin Jacobsson

Department of Microbiology
Swedish University of Agricultural Sciences

Uppsala

Master thesis 2009:1

ISSN 1101-8151
ISRN SLU-MIKRO-EX–09/1–SE

Abstract

In temperate countries such as Sweden, where much rain falls during the harvest period, problems can occur later during preservation of feed grains because of too high water contents in the grains. Drying by heated air is the most commonly used operation in Sweden to reduce water content in grains. If airtight storage is used instead, energy and money can be saved, as airtight storage only consumes 2 % of energy needed for heated air drying.

Through biopreservation, where added microorganisms enhance the stability of the cereals in the airtight storage system by preventing mould growth, a more stable preservation can be obtained. To achieve a safe storage, insensitive to air leakage, the starter culture must work properly independently of the moisture content in the grains. To expand the acceptable region of moisture content, combinations of several organisms can be used as a starter culture. A mixture of the yeast *Pichia anomala* J121, a lactic acid bacteria (LAB) and a propionic acid bacteria (PAB) may be an ideal starter culture, as the yeast can work in relative dry grains and the bacteria in more moist grains. However, having several organisms in the starter culture can cause problems. For example, *P. anomala* J121 can metabolize lactic acid produced by LAB, as oxygen enters the system in the moist grains. This results in an increase in pH, allowing detrimental organisms to grow. The ability of different LAB and PAB isolates to inhibit *P. anomala* J121 was therefore investigated. Slight inhibition of the yeast was only observed for *Propionibacterium freundenreichii* subsp. *shermanii* MP5, *P. freundenreichii* MP23 and *P. thoenii* MP11. To work as a starter culture, the microorganisms must be able to grow and produce organic acids and/or other antifungal compounds. Therefore, we investigated the ability of different combinations of PAB, *Lactobacillus plantarum* MiLAB393(pLV100) and *P. anomala* J121 to inhibit the mould species *Penicillium roqueforti* J5 at moisture contents of 25 % and 33 % in a mini silo system. The mould growth was mainly affected by *P. anomala* J121. Since the yeast seemed to have lower ability to grow at moisture content of 33 %, a starter culture with several organisms seems to be preferred. Of the PAB investigated in the study, *Propionibacteria thoenii* MP11 is the most suitable PAB in a starter culture, due to its ability to grow and produce organic acids. However, additional LAB and PAB need to be characterized and tested in different combinations.

Keywords: Lactic acid bacteria, *Pichia anomala* J121, propionic acid bacteria, moisture content, water activity, starter culture, mould, inhibition, biopreservation, airtight storage

Sammanfattning

I tempererade länder som Sverige, där det faller mycket nederbörd under skördeperioden, kan höga vattenhalter i spannmålen orsaka problem vid lagerhållningen. Varmluftstorkning är den mest använda metoden i Sverige för att reducera spannmålens vattenhalt. Om lufttät lagring skulle användas istället kan energi och pengar sparas, då lufttät lagring endast förbrukar 2 % av energin som krävs vid torkning.

Biokonservering, där tillsatta mikroorganismer ökar kvalitén på lufttät lagrad spannmål genom att inhibera mögeltillväxt, kan ge mer stabila lagringsförhållanden. För att uppnå en säker lagring som är stabil mot syreläckage, är det viktigt att startkulturen fungera oberoende av spannmålens fukthalter. För att öka området för acceptabla fukthalter kan en kombination av flera organismer användas i startkulturen. En blandning av *Pichia anomala* J121, en mjölksyrabakterie (LAB) och en propionsyrabakterie (PAB) skulle kunna vara en ideal startkultur, eftersom jästen är verksam i relativt torra spannmål och bakterierna i mer fuktiga spannmål. Problem kan dock uppstå då flera organismer ingår i startkulturen. Till exempel kan *P. anomala* J121 metabolisera mjölksyra, producerat av LAB i fuktigt spannmål, då syre blir tillgängligt i systemet. Detta resulterar i stigande pH-värde, vilket tillåter skadliga organismer att växa. Följaktligen studerades olika LAB och PAB isolats förmåga att inhibera *P. anomala* J121. Marginell inhibering av jästen inträffade enbart för *Propionibacterium freundenreichii* subsp. *shermanii* MP5, *P. freundenreichii* MP23 och *P. thoenii* MP11. För att vara en fungerande startkultur måste mikroorgansimerna ha förmåga att växa och producera organiska syror och/eller andra hämmande föreningar. Därför undersöktes förmågan att inhibera mögelarten *Penicillium roqueforti* J5 vid fukthalterna 25 % samt 33 % i ett minisilosystem för olika kombinationer av PAB, *Lactobacillus plantarum* MiLAB393(pLV100) och *P. anomala* J121. Mögeltillväxten påverkades i huvudsak av *P. anomala* J121. Eftersom jästen tycktes ha en sämre förmåga att växa vid en fukthalt av 33 %, tycks en blandning av flera organismer vara att föredra. Av de undersökta PAB, var *P. thoenii* MP11 mest lämpad att ha i en startkultur, på grund av dess förmåga att växa och producera organiska syror. Fler olika kombinationer av tillsatta LAB och PAB bör dock studeras för att en väl fungerande startkultur ska kunna sättas samman.

Nyckelord: Mjölksyrabakterier, *Pichia anomala* J121, propionsyrabakterier, fukthalt, vattenaktivitet, startkultur, mögel, inhibering, biokonservering, lufttät lagring

Contents

1. Aim of the study

The aim of this project was to identify lactic acid bacteria that can be included in a new starter culture together with *Pichia anomala* J121 that would prevent growth of spoilage organisms such as mould, regardless of the moisture content of the grains. During the study, the effects of adding different propionic acid bacteria in the starter culture were also investigated.

2. Introduction

In total, Swedish farmers produce approximately five million tons of cereal grains every year (SJV, 2008). Approximately 10 % of the crops are spoiled postharvest every year, due to growth of detrimental organisms caused by an incomplete storage system (Aidoo, 1993). Today, a common method to preserve feed in Sweden is drying. Since drying consumes much energy it is important to find alternative methods for grain storage. Airtight storage is a method that consumes less energy than drying (Druvefors, 2004). Biopreservation, where microorganisms are added to the airtight storage system, enhance the stability of the preservation due to their inhibitory effect on spoilage organisms.

2.1 Spoilage organisms on grains

The natural microflora of the grains consists of microorganisms such as moulds, yeasts and bacteria and usually changes from pre- to postharvest. Generally, bacteria that are found on grains are non pathogenic e.g. different LAB species. Pathogenic bacteria as *Salmonella* species and *Escherichia coli* can sometimes be found on grains, due to their contact with birds and rodents (Hocking, 2003). Olstorpe concluded that the yeast flora of grain can vary. Yeast species found at harvest are e.g. different *Auerobasidium pullulans* and *Cryptococcus wierinage* and after storage species as *C. wierinage* and *Pichia anomala* can be found (Olstorpe *et* al., 2008). Moulds that are common in field are *Alternaria*, *Fusarium*, *Aspergillus* and *Penicillium*. All of them have the ability to produce mycotoxins before or in the beginning of the storage period (Hocking, 2003). Since *Alternaria* and *Fusarium* have lower ability to grow and produce mycotoxins in dry grains, *Aspergillius* and *Penicillium* are regarded as a larger problem during storage (Hocking, 2003).

One of the most common spoilage organisms on grains during airtight storage is *Penicillium roqueforti*, due to its ability to grow at low temperature, low partial pressure of O_2 and high carbon dioxide concentration. *P. roqueforti* also has the potential to produce mycotoxin (Petersson *et* al. 1999). When this spoilage organism grows, the metabolic activity raises the temperature and the moisture content in the grains. This allows organisms, needing higher water concentration, to proliferate. Other effects of mould growth are for example losses in dry matter, discoloration, reduction in nutritional value and production of mycotoxins (Wienberg & Muck, 1996). Spoilage of stored feed can be prevented by a proper preservation technique. One primary goal, to receive a safe preservation, is to reduce the water activity.

2.2 Water activity

The water activity (a_w) is a measure of the amount of water present in the material in free form. Microorganisms require water in free form for their growth and for survival, which makes water activity to an important factor during preservation. Water activities are estimated by calculating the quotient of the vapor pressure over the material and over distilled water (Thougaard *et* al., 2001). When $a_w < 0.7$, equal to 14 % moisture of the material, growth of spoilage fungi are inhibited (Petersson, 1998). One way to reduce the amount of free water in the material is drying. If there are difficulties in keeping $a_w < 0.7$ some mould species can start to grow. During growth, they convert glucose through their

respiration to carbon dioxide and water. Hence the total amount of water in the material rises. Higher a_w allows more spoilage species to grow (Petersson, 1998).

There are huge problems for Swedish farmers to harvest the grains at desired moisture content, since there are big variations in temperature and amount of precipitation during the harvest period. Different preservation techniques can be used to achieve a safe preservation in spite of the variations in moisture content, e.g. drying can be used.

2.3 Preservation by drying

Around 75 % of the grains harvested in Sweden are preserved with the energy demanding process, drying (rev. Druvefors, 2004). There are three different ways of drying grains; cold air, cold air with additional heating and hot air. Drying with hot air gives the cereals more homogenous and lower moisture content than drying with cold air does. The cereals are normally harvested at a water content of 20-22 %, and need to be dried to a water content of 13 % ($a_w < 0.65$) to achieve a safe preservation. Drying the harvest with hot air to this level cost much energy, approximately 60 % of the energy consumed by the farms. Considering that the energy price had increased a lot during the last decade (SCB, 2007) it is important to find a stable alternative storage system, which consumes less energy. Less usage of energy is also beneficial for the environment. (rev. Olstorpe, 2008)

2.4 Preservation by airtight storage

Airtight storage is an alternative to drying the cereal grains. It saves energy and money, since it only consumes approximately 2 % of the energy required for hot air drying (Table 1). Only grains used for animal feed could be stored this way, since airtight storage has negative effects on gluten protein and germination capacity. In spite of this, a lot of money could be saved by using the airtight storage system, since 60 % of the harvested grains in Sweden are used as animal feed. (Druvefors, 2004)

Table 1 Energy consumption (kW/ha) of different preservation system (Druvefors, 2004).

	Moisture content at harvest		
Method	20 %	22 %	25 %
Heated air drying	665	885	1295
Airtight storage	15	15	15

Higher water contents than what is used during preservation by drying are allowed in airtight storage, still enabling a safe storage. The process of airtight storage is built on a closed system with anaerobic conditions, caused by respiration by the endogenous microorganisms on the grains (Druvefors *et* al., 2002). An anaerobic environment inhibits growth of aerobic spoilage organisms. A common problem encountered in airtight storage is leakage, which modifies the conditions of the closed system. Oxygen can enter and allow growth of aerobic spoilage organisms in the closed system. Also temperature variations in the surrounding environment and feed outtake may result in increased oxygen concentrations (Petersson *et* al., 1999). During long time storage, spring and summer can be critical periods during the preservation. Large variations in temperature between days and nights, that cause pressure fluctuations, may bring on air flow into or out of the system (Druvefors *et* al., 2002). Using biopreservation, where a starter culture

consisting of microorganisms is added to the grains, may enhance the stability of airtight storage, as the added microorganisms can reduce the time required to reach stable conditions and also have inhibitory effect on spoilage organisms.

2.5 Biopreservation

In biopreservation, a starter culture of microorganisms is added to a system to inhibit spoilage organisms, without affecting the quality of the original product (Thougaard *et* al., 2001). The starter culture could besides inhibiting detrimental organisms also enhance the quality of the product, either by prolonging the durability or bring on a specific texture or color of the product. Adding a non-indigenous species to a natural ecosystem can be discussed. Some think it is harmful, as the biocontrol agent can destroy the natural ecosystem, while others find it more ecofriendly than adding chemical agents. Industries that use biopreservation are producers of fruits, vegetables and other food stuff as well as flowers and animal feed. The storage system should be closed and by manipulating e.g. temperature and moisture content, optimal condition for the biocontrol organism can be reached (Druvefors, 2004).

Problems of determining the mechanism behind the biocontrol agent are rife, since it is difficult to perform experiments that exclude all other potential mechanisms but one. Competition for nutrients, production of organic acids or other metabolites and parasitism are examples of biocontrol mechanisms. In most cases, the possibility that more than one mechanism is involved is likely. (Druvefors, 2004)

A starter culture can consist of one microorganism, or of several microorganisms that co-operate to improve the preservation (Thougaard *et* al., 2001). The use of more than one biocontrol organism may allow more variations in grain quality as different organisms may grow differently under different conditions, such as moisture contents or temperatures. Having several organisms in the starter culture can also cause problems, e.g. one agent can metabolize the metabolic product from the controlling organism under certain conditions. Hence the inhibiting environment can change so detrimental organisms can grow. Organisms that can be used as a biocontrol agent are e.g. lactic acid bacteria (LAB), yeast and propionic acid bacteria (PAB) (Merry & Davies, 1999; Petersson *et* al., 1999).

2.5.1 *Pichia anomala* J121 as a biocontrol agent

The yeast *Pichia anomala* J121 does not produce mycotoxins or allergenic spores and it is not pathogenic to human and therefore valid for use as a biocontrol agent. Also its ability to grow at low water activity ($a_w = 0.85$), low partial pressures of O_2, temperatures between 3˚C-37˚C, at pH between 2.0-12.4 and use different substrates make it useful (Petersson, 1998). *P. anomala* J121 can grow live under anaerobic condition, despite being an aerobic organism. During its anaerobic fermentation products as ethanol, acetic acid and ethyl acetate are produced besides water, carbon dioxide and biomass, which also are produced during its respiration. Another major profit of the yeast is that it can tolerate many of the pesticides that are used in the fields before harvest. Positive effects on both food and feed can also occur after addition of the yeast, since yeast cells contain high amounts of vitamins, minerals and essential amino acids (rev. Druvefors, 2004).

Metabolic products of *P. anomala* J121 with mould inhibitory effects are ethyl acetate and ethanol (Druvefors *et* al., 2005). Studies made with *P. anomala* J121 demonstrates its ability to reduce growth of *Penicillium roqueforti* in leaking airtight storage systems, both small and large scale, at high moisture levels (Druvefors *et* al., 2002; Peterson *et* al., 1999). It has been concluded that competition for space and nutrient, as sugar and nitrogen, not alone can explain the antifungal activity of *P. anomala* J121 (Druvefors *et* al., 2005).

P. anomala J121 is not always positive for food and feed preservation. As air enters the system the yeast can metabolize lactic acid and instead produce the less strong acid, acetic acid. As pH increases, detrimental microorganisms can grow (Middelhoven & van Balen, 1988). Fungal metabolism also results in a higher level of available water further promoting growth of unwanted microorganisms.

2.5.2 Lactic acid bacteria as a biocontrol agent

Using LAB as biocontrol agents is not a new invention. The food and feed industry have used the natural microflora of LAB in different products for centuries. For example, soured milk can be produced by the LAB present in milk. The function of LAB in food production differs from feed production. Feed production uses LAB primarily as biocontrol agents for example to improve the ensiling process. In food production LAB can also be used to obtain a specific flavor or surface of the product. Other positive effects of LAB is that they are presumed to be harmless and works in some cases as probiotics in human and animal health. (Schnürer & Magnusson, 2005)

LAB are gram-positive, non spore producing, anaerobic bacteria. The bacteria can survive, and sometimes also grow, in aerobic environments, since they are aero tolerant anaerobes (Madigan & Martinko, 2006). LAB need several nutrients such as fermentable carbohydrates, amino acid, fatty acids, salts and vitamins to survive. Hence they are found in nutrient rich environments. Depending on the metabolism, LAB can be classified in three different groups; homo-, heterofermentative and facultative (Ström, 2005). The homofermentative LAB, which mainly produce lactic acid, uses the glycolysis pathway and the heterofermentative produce lactic acid, carbon dioxide and acetic acid using the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway. Facultative LAB can use both the glycolysis and the 6-PG/PK pathway. Which one that is used depends on the carbon source. If hexos fermentation is used, the faculative LAB will act as homofermenters whereas under pentose fermentation they act as heterofermenters.

Preservation by LAB proceeds owing to its lactic acid production, which reduce pH. As a result of the decline in pH, spoilage microorganisms cannot grow and may even be killed. Homofermentative LAB produces more of the strongest acid, lactic acid, than the heterofermentative. This makes them more suitable as biocontrol agents. Also competition for essential nutrients plays a part when LAB inhibits growth of other microorganisms (Holzer *et* al., 2003). In addition, LAB can produce other antimicrobial substances such as reuterin, which improves the preservation as it has an inhibitory effect on bacteria, yeast and moulds growth. (Ström, 2005)

2.5.3 Propionic acid bacteria as a biocontrol agent

PAB are anaerobic gram positive bacteria. Their fermentation converts lactic acid, carbohydrates and alcohols mainly to propionic acid, acetic acid and CO_2 (Madigan & Martinko, 2006). They usually grow rather slowly. Organic acids that are produced during their metabolism have antifungal activities mainly on moulds but also on yeasts (Lind *et* al., 2005). Their antifungal effect makes them putative biocontrol agents. Studies have showed that PAB could enhance the aerobic stability of silage when sealing is delayed, resulting in a delay of the rapid decline in pH caused by lactic acid production by LAB (Merry & Davies, 1999). This favors growth of PAB. Other positive effects of having PAB in a starter culture are that their acid production can reduce both the ammonia concentration and temperature. They can also stimulate growth of LAB (Merry & Davies, 1999).

2.6 The ensilaging process

In Sweden most farmers rely on natural fermentation caused by the epiphytic LAB present of the crop at harvest. However, chemical and biological additives can be used in the ensilage process to improve the fermentation phase. Biological additives include bacterial inoculants and enzymes. Benefits of biological preservation are that the biological additives have innocuous effect on grains, not pollute the environment and that they are regarded as natural products. During the fermentation phase a decline in pH is obtained due to the acid production, mainly the production of lactic acid by LAB. The decrease in pH inhibits growth of spoilage organisms in the grains as the undissociated form of the acid can pass through their membrane, resulting in a weak growth by extending the lag phase and causing a poor growth in the exponential phase (Lindgren, 2008; Lambert & Stratford, 1999). (Weinberg & Muck, 1996)

Whether the farmers rely on the epiphytic LAB or use a defined biological additive, the fermentation process starts at a moisture content of 30-45 % (Olstorpe *et* al., 2008). A combination of low pH and high level of carbon dioxide in the environment results in a safe preservation of the grains, since growth of spoilage organisms are inhibited. The ensilage process can be divided into four phases (Weinberg & Muck, 1996):

- **Aerobic**- oxygen is present enabling growth of aerobic organisms, pH 6.0-6.5.
- **Fermentation**- oxygen has been consumed by aerobic microorganisms and plant respiration. LAB and other anaerobic bacteria can grow. pH decreases to 3.8-5.0 due to the production of organic acids by the anaerobic bacteria.
- **Stable**- low activity of microorganisms.
- **Feed out**- the system is exposed to air during removal of feed. Aerobic organisms as yeasts and moulds can start to grow.

2.6.1 Problems during the ensilage process

Long time storage exposes the storage system to large variations in the surrounding temperature and the atmospheric pressure (Druvefors *et* al., 2002). This causes problems such as air flow through the shell during the fermentation and the stable phases. Other things that can cause problems during the ensilage process are the quality of the grains

and incorrect sealing. If the sealing is leaking, flow of oxygen in to the system is possible, which allows growth of aerobic microorganisms that are in the system.

Delayed sealing can also be a problem. When the sealing is delayed the aerobic phase is prolonged. This can result in a reduction of LAB fermentable substrate, e.g. water soluble carbohydrate. Combinations of incomplete pH reduction and high moisture of the grains can result in growth of clostridia that ferment lactic acid causing an increase in pH, due to their production of butyric acid. Also loss in dry matter occur (Weinberg & Muck, 1996). The bacterium *Clostridium botulinum* has the ability to produce botulinum toxin. The toxin is the most potent neurotoxin known and can cause muscles paralysis (Madigan & Martinko, 2006). *C. tyrobutyricum* can also be found in silage and has the ability to cause late blowing in cheese if present in raw milk. Hydrogen gas, produced during butyric acid fermentation, cause formation of holes in the cheese (Klijn *et* al., 1995).

As air enters the system at feed out phase, aerobic organisms as yeast can metabolize lactic acid, hence pH will increase. This enables growth of other, less pH tolerant microorganisms. (Holzer, 2003)

2.7 Problems in finding an ideal starter culture

Different storage systems need diverse moisture contents of the grains to achieve a safe storage. In Sweden, where the unstable weather during the harvest period makes it difficult to harvest at desired water activity, airtight storage with an additive of a starter culture is a useful alternative. This can expand the interval of acceptable moisture contents, as the added organisms could work in a wide region of moisture. Having a mixture of LAB, PAB and *P. anomala* J121 could be a suitable combination of organisms that would work over a wide range of water activities. In wet grains, LAB and PAB would be responsible for the preservation, since their growth and fermentation needs a moisture content of 30-45 % (Olstorpe *et* al., 2008). In dryer grain growth and production of metabolites by *P. anomala* J121 would have a preserving effect. However, one putative problem with this mixture of organisms is that the yeast can start to metabolize lactic acid when moist grains are exposed to air, which can cause an increase in pH allowing growth of other spoilage organisms (Middelhoven & van Balen, 1988). Finding LAB and PAB that can prevent growth of *P. anomala* J121, during feed out phase, are therefore of high interest.

3. Materials and methods

3.1 Bacterial and fungal isolates

Bacteria and fungi from the culture collection at Department of Microbiology, Swedish University of Agricultural Sciences in Uppsala, were used in this study. Studied microorganisms were different lactic acid bacteria, propionic acid bacteria, *Pichia anomala* J121 and *Penicillium roqueforti* J5. Investigated LAB had been isolated from crimped barley stored in plastic tubes (Olstorpe *et* al., 2008). The *Lactobacillus plantarum* MiLAB393(pLV100), a chloramphenicol resistant transformant of the well characterised antifungal strain *L. plantarum* MiLAB393, were aslo studied (Ström *et* al., 2005; Broberg *et* al., 2007). Analysed PAB were isolated from various sources (Helena Lind, personal contact). The mutant *Propionibacterium thoenii* MP028RS02 (Kind gift from Helena Lind), a spontaneous mutant of *P. thoenii* isolated by repeated selection on plates containing streptomycin or rifampicin, were also investigated. Growth conditions for microorganisms are given in Table 2 and 3. The anaerobic environment was created using the GasPak System (BBL, MD, USA).

Table 2 Growth condition for studied microorganisms

Organism	Media	Growth condition
LAB	MRS-agar (Oxoid Ltd., England)	48 h at 30˚C, anaerobic
	MRS-broth (Oxoid Ltd., England)	24 h at 30˚C, anaerobic
PAB	NL-agar (Appendix 1)	96 h at 30˚C, anaerobic
	NL-broth (Grinstead and Barefoot (1992), Appendix 1)	96 h at 30˚C, anaerobic
P. anomala J121	MEA-agar (Oxoid Ltd., England)	48 h at 30˚C, aerobic
	MEA-soft agar (Appendix 1)	48 h at 30˚C, aerobic
	GPY-broth (Appendix 1)	24 h at 30 ˚C, aerobic
P. roqueforti J5	MEA-agar slant (Oxoid Ltd., England)	7 days at 25˚C, aerobic

Table 3 Composition of the selective agar plates used for quantification of different organisms.

Medium	Composition	Studied organism
MRS-CD	MRS-agar (Oxoid Ltd., England) Chloramphenicol [0.15 µg/ml] (Boehringer Mannheim, Germany) Delvocid®[0.1 g/l] (DSM Food Specialties, The Netherlands)[1)]	*L.plantarum* MiLAB393(pLV100)
MEA-C	MEA-agar (Oxoid Ltd., England)	*P. anomala* J121

	Chloramphenicol [0.1 mg/ml] (Boehringer Mannheim, Germany)[2)]	
MEA-CC	MEA-agar (Oxoid Ltd., England)	*P. roqueforti* J5
	Chloramphenicol [0.1 mg/ml] (Boehringer Mannheim, Germany)	
	Cycloheximide [10 µg/ml] (Sigma-aldrich, United Sates)[3)]	
NL-RSD	NL-agar (Appendix 1)	*P. thoenii* MP028RS02
	Rifampicin [40 µg/ml] (DUCHEFA, The Netherlands)	
	Streptomycin [400 µg/ml] (Merck, Germany)	
	Delvocid®[0.1 g/l] (DSM Food Specialties, The Netherlands)	

1) Delvocid® contains Natamycin that inhibits fungal growth.
2) 0.1 mg/ml chloramphenicol inhibits growth of bacteria.
3) 10 µg/ml cycloheximide has earlier been shown to inhibit growth of *P. anomala* J121 but not *P. roqueforti* J5 (Björnberg & Schnürer, 1993).

3.2 Inhibition of P. anomala *J121 by LAB and PAB*

The overlay-method described by Magnusson and Schnürer (2001) was used to determine the capacity of LAB and PAB to inhibit *P. anomala* J121. A total of 157 LAB and 34 PAB isolates were investigated.

P. anomala J121 was grown in 25 ml GPY-broth (Appendix 1) on a rotary shaker at 150 rpm over night at 30˚C. After incubation, yeast cells were counted in a Bürker counting chamber and diluted with peptone water (Appendix 1) to reach a concentration of 10^5 cells/ml. To verify the concentration, 100 µl portions of serially diluted yeast cells were plated on MEA-agar.

Fresh colonies of LAB, grown for 48 h on MRS-agar, or PAB, grown for 96 h on NL-agar, were resuspended in peptone water to an optical density corresponding to McFarland 2. Parallel lines, each 2 cm long, were streaked on MRS- or NL-agar (Figure 1) and plates were incubated as before. One ml containing 10^5 cells of *P. anomala* J121 /ml was mixed with 9 ml MEA-soft agar and poured onto the agar plates. Plates were incubated for 48 h at 30˚C under aerobic condition. Inhibition effect was graded according to the size of the clear zone surrounding the lines according to Magnusson and Schnürer (2001). Investigations of a synergistic inhibition effect with a mixture of PAB and *L. plantarum* MiLAB393 (pLV100), each resuspended to McFarland 2, were also performed.

Figure 1 MRS-agar plate with parallel lines of LAB (Photo: Malin Larsson, 2008).

3.3 *Growth of* L. plantarum *MiLAB393(pLV100),* P. anomala *J121 and* P. thoenii *MP028RS02 in mini silo*

The ability of *L. plantarum* MiLAB393(pLV100), *P. anomala* J121 and *P. thoenii* MP028RS02 to grow in wheat with the moisture content of approximately 20 % and 30 % was investigated. Unsterile, dry wheat (cv. Kosack) from Lantmännen, Uppsala Sweden, stored at room temperature was hydrated with de-ionized water to a moisture content of 20 % and 30 % respectively. The wheat was incubated at 2˚C for 72 hour to homogenize the moisture. Since the wheat was not sterilized before the experiment, bacterial and fungal growth was studied as described below.

3.3.1 Inoculation of mini silos

L. plantarum MiLAB393(pLV100), *P. anomala* J121 and *P. thoenii* MP028RS02 were grown as earlier described, mixed in a final volume of 5 ml peptone water and added to 500 g soaked wheat in a glass jar to reach the concentrations shown in Table 4. An even distribution of the organisms was obtained by shaking the jars.

Table 4 Concentration of added bacteria and yeast into soaked wheat.

Species	**Concentration (CFU/g)**
L. plantarum MiLAB393 (pLV100)	10^5
P. thoenii MPO28RS02	10^6
P. anomala J121	10^5

Approximately 17 g of inoculated grains were added to thick walled test tubes, which were sealed with a rubber stopper (Figure 2). The rubber membrane was perforated with a needle to enable gas release and to simulate an air leakage. Silos were incubated at 25 °C for two weeks. Two replicates for each treatment were done.

Figure 2 Mini silos with soaked wheat with a mixture of bacteria and yeast. The mini silo to the left was soaked to moisture content of 20% and the right was soaked to moisture content of 30% (Photo: Malin Larsson, 2008).

3.3.2 Quantification of bacteria and yeasts

The number of viable bacteria and yeasts were determined at 0 h, 2 h, 4 h, 6 h, 12 h, 24 h, 48 h, 96 h, 1 week and 2 week after inoculation. At each occasion, wheat from two mini silos was each diluted ten-fold in 0.9 % NaCl (Merck, Germany) and 0.01% Tween 80 (Merck, Germany). The mixtures were homogenized for 2 minutes at normal speed in a Stomacher 400 (Seward, UK). Growth was determined as CFU/g by using selective agar plates (Table 3) and the mean of the two replicates was then calculated.

3.3.3 Determination of water activity, moisture content and pH

Water activity and moisture content were analysed before and after addition of organisms. The water activity of the grains was measured at room temperature using a CX-2 AquaLab instrument (Decangon Devices, Washington, USA). Moisture content was determined by comparing the weight before and after drying 10 g of wheat at 103˚C for 16 h. The pH of the homogenate was determined by using a Metrohm 632 pH-meter (Metrohm, Switzerland)

3.3.4 Determination of organic acids

High pressure liquid chromatography (HPLC) was used to determine the concentrations of organic acids in the inoculated wheat. One ml of the homogenate was sterile filtrated through a 0.45 μm pore size filter (Sarstedt, Germany) and stored at -20°C until used. Five μl of each sample were injected to an Aglient 1100 series HPLC (Aglient Technologies, US) equipped with a ReZex ROA-Organic Acid H+ column (300 x 7.8 mm) and a refractive index detector. Five mM H_2SO_4 was used as mobile phase at flow rate of 0.6 ml/min at temperature 60˚C.

3.4 Inhibition of P. roqueforti J5 *in mini silos*

The ability of different combinations of *L. plantarum* MiLAB393 (pLV100), *P. anomala* J121 and PAB to inhibit growth of *P. roqueforti* J5 was investigated. *P. roqueforti* J5 was grown on three MEA-agar slants in 7 days and spores collected in peptone water.

3.4.1 Inoculation of mini silos

Mini silos were prepared as earlier described using different combinations of microorganisms (Table 5). There were two replicates of each treatment. Microorganisms were mixed in a final volume of one ml and added to 75 g soaked wheat to reach a concentration of *L. plantarum* MiLAB393(pLV100) of 10^5 CFU/g, *P. anomala* J121 of 10^5 CFU/g, PAB of 10^6 CFU/g and *P. roqueforti* J5 of 10^3 CFU/g.

Silos were incubated at 25°C for three weeks and microbial and chemical analysis performed as described earlier. PCR fingerprinting, with the primer $(GTG)_5$, was used to verify counted colonies on the NL-D agar, since other bacteria than PAB can live on the plates. Reaction conditions were initial denaturing at 95°C for 7 min followed by 29 cycles of 90°C for 30 s, 95°C for 1 min, 40°C for 1 min and 65°C for 4 min, ending with a step of 65°C for 16 min. The PCR-product were applied on a 1 % agarose gel in 0.5 x TBE buffer and run at 80 V, 60 mA for 3 h. The gel was supplemented with ethidium bromide to visualise the PCR-products.

Table 5 Composition of added bacteria, yeast and mould into soaked wheat.

Treatment	**Microorganism**
1	*P .roqueforti* J5
2	*P. roqueforti* J5, *L. plantarum* MiLAB393(pLV100), *P. anomala* J121,
3	*P. roqueforti* J5, *L. plantarum* MiLAB393(pLV100)
4	*P. roqueforti* J5, *P. anomala* J121
5	*P. roqueforti* J5, *L. plantarum* MiLAB393(pLV100), *P. anomala* J121 *P. thoenii* MP028RS02
6	*P. roqueforti* J5, *P. anomala* J121, *P. thoenii* MP028RS02
7	*P. roqueforti* J5, *L. plantarum* MiLAB393 (pLV100), *P. anomala* J121, *P. freundenreichii* subsp. *Shermanii* MP5
8	*P. roqueforti* J5, *P. anomala* J121, *P. freundenreichii* subsp. *Shermanii* MP5
9	*P. roqueforti* J5, *L. plantarum* MiLAB393 (pLV100), *P. anomala* J121 *P. freundenreichii* MP23
10	*P. roqueforti* J5, *P. anomala* J121, *P. freundenreichii* MP23
11	*P. roqueforti* J5, *L. plantarum* MiLAB393 (pLV100), *P. anomala* J121, *P. thoenii* MP11
12	*P. roqueforti* J5, *P. anomala* J121, *P. thoenii* MP11

4. Results and Discussion

4.1 Inhibition of P. anomala *J121 by LAB and PAB*

It is difficult to harvest grains at the water activity required to achieve a safe preservation. A good starter culture could expand the acceptable variation in water activity, if it would prevent growth of spoilage organisms in both relatively dry grains and in more moist grains. It has earlier been shown that *Pichia anomala* J121 has the ability to prevent growth of detrimental organisms on grains with a water activity of approximately 0.95 (Petersson *et* al., 1999; Druvefors & Schnürer, 2005). A screening of moist crimped grains showed that lactic acid bacteria had the ability to constrain growth of spoilage organisms in grains with a moisture content of approximately 30 % (Olstorpe *et* al., 2008). Spontaneous fermentation by lactic acid bacteria starts at this moisture content. A mixture of *P. anomala* J121 and LAB may be an ideal starter culture that works over a wide range of different water activities. Notable are that yeasts compete with LAB for the same substrate, and also has the ability to metabolize lactic acid in moist grains during feed out phase, resulting in an increase in pH (Middelhoven & van Balen, 1988). Therefore, we decided to screen a collection of LAB isolated from stored crimped grains to identify strains that would inhibit growth of *P. anomala* J121 in moist grains. Propionic acid bacteria were also included as they produce propionic and acetic acid, which are known to inhibit yeast growth (Merry & Davies, 1999).

None of the studied LAB had the capacity to inhibit *P. anomala* J121. An example of a typical result is shown in Figure 3. For PAB, the inhibition was not strong enough to give clear zones, which made quantification of the inhibition impossible. However, slight inhibitions of *P. anomala* J121 were observed between or over the lines of *Propionibacterium freundenreichii* subsp. *shermanii* MP5, *P. freundenreichii* MP23 and *P. thoenii* MP11.

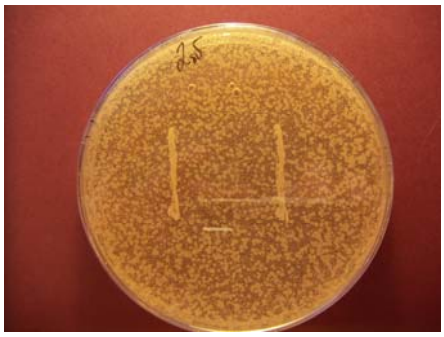

Figure 3 LAB overlayed with *P. anomala* J121 (Photo: Malin Larsson, 2008)

Using a mixture of PAB and *Lactobacillus plantarum* MiLAB393(pLV100) did not result in a stronger inhibition of *P. anomala* J121 than the corresponding PAB alone. These results are in conflict with a study made by Schwenninger and Meile (2004), where a synergetic effect between LAB and PAB was detected. The mixture with *L. paracasei* subsp. *paracasei* and PAB showed a greater inhibition of yeast found in dairy products, than the corresponding bacteria alone. Greatest synergetic effect was observed at 6°C. As the temperature rose, lower synergetic effect was observed. In the present study the

mainly homofermentative *L. plantarum* was used compared to the facultative heterofermative *L. paracasei* subsp. *paracasei* in the study by Schwenninger and Meile. Thus, differences in lactic acid production and temperature may explain the different results. The absence of the synergetic effect in this study could be caused by the *L. plantarum*, which grows more rapidly than PAB and produce a large amount of lactic acid. Former investigations showed that PAB are sensitive to a rapid decline in pH that is caused by production of lactic acid by LAB (Merry & Davies, 1999).

A study of a starter culture with both LAB and PAB are, in spite of this, of interest. In the early stage of the preservation, the bacteria from the added culture can grow independently of one another. This can result in a production of propionic acid before PAB are inhibited by the LAB. Adding a higher number of PAB than LAB in the starter culture can result in a higher quantity of propionic acid before the PAB are out competed by the LAB. Since propionic acid and acetic acid have higher pK_a values than lactic acid (Table 6), they will have a larger fraction of the undissociated form at a certain pH, according to the Henderson-Hasselbach equation $pH = pK_a + \log([A^-]/[HA])$. Only the undissociated form of the acid can pass through the membrane causing an inhibition of the growth, by extending the lag phase and causing a poor growth in the exponential phase (Lindgren, 2008; Lambert & Stratford, 1999). If PAB produce propionic acid and acetic acid before their growth is inhibited by the decrease in pH mediated by the production of lactic acid by LAB, a synergetic effect may be obtained which would enhance the preservation. Therefore, we decided to do further study with mixtures of LAB and PAB.

Table 6 pK_a values for propionic acid, lactic acid and acetic acid (Lind *et* al., 2005).

Acid	pK_a
Propionic acid	4.87
Lactic acid	3.86
Acetic acid	4.76

4.2 Growth of L. plantarum *MiLAB393(pLV100),* P. anomala *J121 and* P. thoenii *MP028RS02 in mini silos*

To be useful in a starter culture, the microorganisms must be able to survive and grow in the grains. Therefore, we investigated the growth of *L. plantarum* MiLAB393(pLV100), *P. anomala* J121 and *Propionibacterium thoenii* MP028RS02 in a mini silo system at two different moisture contents. Even though the wheat was not sterile, it contained 10^2 CFU/g or less of yeasts and moulds. Microorganisms were added to the wheat at much higher concentrations and therefore the natural microflora on the grains could be neglected. Desired moisture contents and water activities in the wheat were not achieved (Table 7) and consequently the difference between the treatments was less then desired.

Table 7 Water activity and moisture content before and after addition of bacteria and yeast. Data represents mean value (n=2).

Desired moisture	a_w	Moisture content (%)
20 % Before addition of bacteria and yeast	0.965	23.9
20 % After addition of bacteria and yeast	0.954	24.9
30 % Before addition of bacteria and yeast	0.995	30.7
30 % After addition of bacteria and yeast	0.990	30.5

4.2.1 Growth of bacteria and yeasts

L. plantarum MiLAB393(pLV100) grew fastest at the higher moisture content. In the treatment with the moisture content of 31 %, *L. plantarum* MiLAB393(pLV100) started to grow immediately while they seemed to decline in number during the first 12 h in the treatment with the moisture content of 24 % (Figures 4 and 5). However, whether this is true it needs to be investigated further. After two weeks of preservation, the final numbers were 3.5×10^7 CFU/g for the treatment with a moisture content of 24 % and 7.3×10^8 CFU/g for the one with a moisture content of 31 %. This is in agreement with previous results which showed that LAB grew in larger amounts in cereals with higher moisture (Olstorpe *et* al., 2008).

P. thoenii MP028RS02 showed a weak increase in CFU/g in wheat with a moisture content of 31 % (Figure 4). The fastest growth was between days 2 and 7. During the first week, there seemed to be a small decrease of *P. thoenii* MP028RS02 in wheat with a moisture content of 24 % (Figure 5). After two weeks of preservation the CFU/g was stabilized and even a slight increase was detected. Final concentrations in CFU/g for the PAB were 2.2×10^6 in the treatment with moisture of 24 % and 1.1×10^8 in the more moist wheat at the end of the study.

Growth of *P. anomala* J121 was better at the lower moisture content. At 24 % moisture the number of viable cells increased from 1×10^4 CFU/g to 3.2×10^7 CFU/g (Figure 4). This is in agreement with earlier results obtained from studies made in both small and large scale silos (Petersson & Schnürer, 1995; Druvefors, 2004). Inhibition of *P. anomala* J121 by the *L. plantarum* MiLAB393(pLV100) and *P. thoenii* MP028RS02 present was not observed. Also in the treatment with more moist wheat, an increase of *P. anomala* J121 was detected (Figure 5). However, the increase was not as distinct as in the wheat with lower moisture content. It should be noted that 1×10^4 CFU/g of *P. anomala* J121 was added to the moisture content of 24 % compared to 5×10^4 in the wheat with a moisture content of 31 %. It is likely that the difference after two weeks might have been even bigger if the starting concentration had been the same.

A clear difference in decrease of pH was found between the treatments. The higher moisture content, an earlier and a more rapid decrease in pH was observed (Figure 4 and 5). After two weeks of preservation the pH was 5.6 (treatment 24 %) compared to 4.2

(treatment 31%). This huge difference can be correlated to the formation of lactic acid (Table 8). In the wheat with the higher moisture content, lactic acid was detected already after 24 h. After two weeks the concentration had increased tenfold. Also other acids, acetic acid, propionic acid and succinic acid were detected after 2 weeks. In wheat with a moisture content of 24 % small amounts of organic acids, as acetic acid and lactic acid, were detected after 2 weeks of preservation. Notable is that propionic acid was detected in the treatment with a moisture content of 31 % despite the rapid decline in pH caused by the production of lactic acid. A rapid decline in pH has been confirmed to have a negative effect on growth of PAB (Merry & Davies, 1999).

Since all added microorganisms had the ability to survive and grow under conditions given in the mini silos, a second study was made to study their ability to inhibit growth of the spoilage organism *P. roqueforti* J5.

Table 8 Concentrations of organic acids formed during 14 days of preservation. Data represents mean value (n=2).

		Concentration (mg/g dry matter)			
Preservation time	**Moisture content (%)**	**Acetic acid**	**Lactic acid**	**Propionic acid**	**Succinic acid**
0-12 h	24	-*	-	-	-
	31	-	-	-	-
24 h	24	-	-	-	-
	31	-	0.63	-	-
48 h	24	-	-	-	-
	31	-	1.03	-	-
96 h	24	-	-	-	-
	31	-	1.62	-	-
1 week	24	-	-	-	-
	31	1.11	2.84	0.75	-
2 week	24	1.08	1.14	-	-
	31	1.88	6.55	1.26	0.48

* No acid detected

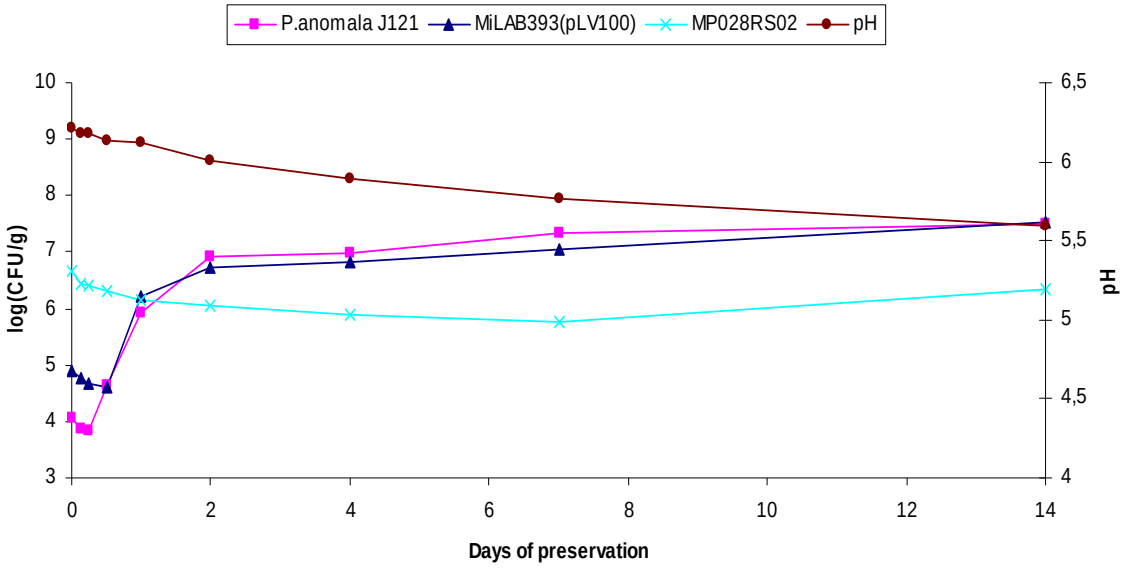

Figure 4 Growth of *L. plantarum* MiLAB393 (pLV100) (—▲—), *P. thoenii* MP028RS02 (—x—) and yeast (—■—) in the mini silo with a moisture content of 24 %. The (—●—) line shows the change in pH. Data represents mean value (n=2).

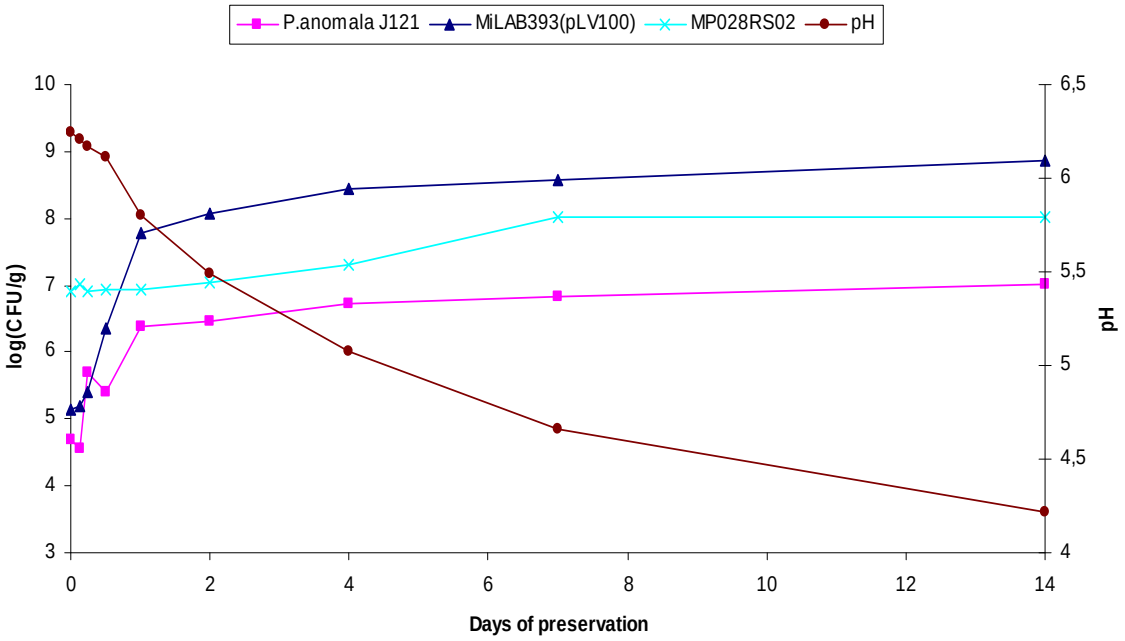

Figure 5 Growth of *L. plantarum* MiLAB393 (pLV100) (—▲—), *P. thoenii* MP028RS02 (—x—) and yeast (—■—) in the mini silo with a moisture content of 31 %. The (—●—) line shows the change in pH. Data represents mean value (n=2).

4.3 Inhibition of P. roqueforti *J5 in mini silos*

To work as a starter culture, the microorganisms must be able to grow and produce organic acids and/or other antifungal compounds. Therefore, we investigated the ability of different combinations of *L. plantarum* MiLAB393(pLV100), *P. anomala* J121 and PAB to inhibit *P. roqueforti* J5. Similar to in the study described above, the desired difference between the moisture contents was not achieved (Table 9). Notable was that the instrument for the water activity analysis not was correctly calibrated.

Table 9 Water activity and moisture content after addition of starter culture. Data represents mean value (n=2).

Desired moisture	a_w	Moisture content (%)
20 %	0.933	24.6
30 %	0.988	32.8

4.3.1 Growth of bacteria, yeasts and mould

Mould growth was only visible in treatments 1 and 3, and then only at the very top of the silos. This is in agreement with the results presented in Table 10, which shows that the highest amounts of moulds were detected in these two treatments. This suggests that a starter culture with only *L. plantarum* MiLAB393 will not be able to inhibit mould growth at these moisture levels. The decline in pH and the amount of lactic acids that were produced (Table 11) was not enough to prevent mould growth in treatment 3. Also the presence of oxygen, as a result of the imperfect sealing, can cause an oxidation of the lactic acid that can allow growth of spoilage organisms in grains. Olstorpe *et* al. (2008) concluded, after analyzing the microbial changes during storage of moist crimped cereal grains, that a high number of LAB cells decreased the growth of spoilage organisms. The dissimilarity between the studies could be caused by the difference of dominant LAB species. In present study, a homofermentative LAB was used, that mainly produces lactic acid resulting in a rapid decline in pH. In the study made by Olstorpe the obligate heterofermentative *Lactobacillus fermentum* was dominant. A heterofermentive LAB produce less lactic acid during their metabolism compared to the homofermentative LAB, resulting in a slow and not as significant decline in pH. *L. fermentum* also produce acetic acid. It is also possible that the microorganisms may behave differently in crimped and intact grains as nutrients probably are more readily available in the former.

In general, mould growths were inhibited to below or near the detection level (10^2) in all other treatments (Table 10). A likely reason for the decrease in number of viable *P. roqueforti* J5 was the growth of *P. anomala* J121. At the lower moisture content *P. anomala* J121 alone is probably responsible for the inhibition of mould growth. The largest numbers of yeast cells were observed at this level (Table 10) and the acid production by *L. plantarum* MiLAB393(pLV100) and PAB was lower than in the more moist grains (Table 11). Earlier studies had already concluded that *P. anomala* J121 effectively reduce the number of *P. roqueforti* J5 cells in the grains at a moisture content around 25 % (Druvefors & Schnürer, 2005; Petersson *et*. al, 1999). The results from treatment 4, where *P. anomala* J121 was added alone also confirmed that it can reduce

mould growth at higher moisture content, though *P. anomala* J121 seemed to grow better at lower moisture content (Table 10). The presence of high concentrations of LAB at 33 % moisture appeared to slightly decrease the number of *P. anomala* J121 cells. Greater difference in number of yeast cells between the two moisture contents were observed when *L. plantarum* MiLAB393(pLV100) was included in the starter culture, compared to using a starter culture without *L. plantarum* MiLAB393(pLV100) (Table 10).

As different from in the previous study, almost no growth of PAB was detected. Generally, higher numbers of PAB were detected at 33 % than at 25 % moisture. Only slight increase of the initial number of added PAB cells were detected at 33 % moisture in treatment 12, where *P. thoenii* MP11 and *P. anomala* J121 were used as starter culture, and in both treatments 9 and 10 with *P. freundenreichii* MP23 (Table 10). Of these two, *P. thoenii* MP11 are most suitable to have in a starter culture, due to its acid production (Table 11). In agreement with the previous study, PAB seems to be inhibited by the large amounts of lactic acid that cause a rapid decline in pH at 33 % moisture (Merry & Davies, 1999), since larger number of PAB cells were detected when small or no amounts of lactic acid were present in treatments without *L. plantarum* MiLAB393(pLV100) (Table 10 and 11). According to Table 10 and 11, *P. freundenreichii* subsp. *shermanii* MP5 is not suitable for use in a starter culture, due to its low ability to survive in the system and lack of detectable production of propionic acid. Also bacteria other than PAB could grow on the NL-D agar, since the plates only inhibit fungi due to the addition of Delvocid®. As *L. plantarum* MiLAB393(pLV100) also grew on these plates, the actual number of PAB could be higher than detected, as *L. plantarum* MiLAB393(pLV100) can affect growth of PAB on the agar plates, since they grow much faster and produce lactic acid. No difference in numbers of colonies was observed between NL-D agar and MRS-CD agar for the *L. plantarum* MiLAB393(pLV100). This can result in fallacious CFU/g for the PAB. However, the absence of propionic acid production showed that PAB did not grow well in the mini silos.

It should be notice that the results from this study may not be statistically significant, since the done treatments were only in two replicates. However, a trend of the ability of an organism to grow, produce acids and inhibit mould growth could be obtained. The reasons for not using more than two replicates were the limitation of mini silos available combined with the will to investigate several organisms.

Farmers that use plastic tubes to obtain an airtight system usually extend the aerobic phase to enable gas produced by the organisms in the grains to leak out before the silos are closed (Matilda Olstorpe, personal contact). It has been concluded that extending the aerobic phase in the ensiling process can increase the aerobic stability when a mixture of LAB and PAB was added to wilted grass, since it favours growth of PAB by delaying the drop in pH (Merry & Davies, 1999). Having a method that allows PAB to grow well could favour their production of propionic acid and acetic acid. Also growth of the aerobic organism *P. anomala* J121 could be favoured because of an extended of the aerobic phase. Having a large number of yeast cells in the grains can in addition to the inhibiting effect also contribute to higher levels of vitamins, essential amino acids and minerals (rev. Druvefors, 2004).

According to Swedish guidelines the maximum CFU/g of mould in cereal grains is 10^5 (SJVFS 2006:81). After three weeks of preservation, all treatments had values below or

near this level, also wheat without any biocontrol agent. In previously published study, the CFU/g of mould was over this level (Druvefors & Schnürer, 2005). It should be noted that the agar plates used for mould growth seemed to affect growth. Approximately 30 % less *P. roqueforti* J5 colonies were detected on MEA-CC- agar, than on the MEA-C-agar. Data obtained from the MEA-C plates could not be used as growth of *P. anomala* J121 would inhibit *P. roqueforti* J5 on the plates. However, a comparison of the treatments could be done.

Table 10 Growth of *L. plantarum* MiLAB393(pLV100), PAB, *P. anomala* J121 and *P. roqueforti* J5 in the inoculated wheat. Data represents mean value (n=2).

Time of preservation	Treatment	Moisture content (%)	log(CFU/g)			
			L. plantarum MiLAB393(pLV100)	*P. anomala* J121	PAB	*P. roqueforti* J5
0 h	1	25	-*	-	-	2.50
		33	-	-	-	2.67
0 h	2-12	25	4.75▪	4.26•	6.09▲	2.77
		33	5.20▪	4.41•	6.33▲	2.70
3 Weeks	1	25	-	-	-	4.97
		33	-	-	-	3.92
3 Weeks	2	25	6.90	7.82	-	<2.00
		33	7.35	6.23	-	<2.00
3 Weeks	3	25	7.24	-	-	4.98
		33	8.17	-	-	5.00
3 Weeks	4	25	-	7.73	-	<2.00
		33	-	6.64	-	<2.00
3 Weeks	5	25	7.15	7.68	5.20	<2.00
		33	8.52	5.66	6.13	<2.00
3 Weeks	6	25	-	7.81	4.39	<2.00
		33	-	6.53	6.17	<2.00
3 Weeks	7	25	7.58	7.63	3.15	<2.00
		33	8.42	6.81	<2.00	2.30
3 Weeks	8	25	-	6.69	<2.00	<2.00
		33	-	6.60	6.60	<2.00
3 Weeks	9	25	5.81	6.77	3.07	2.00
		33	7.39	5.16	6.84	<2.00
3 Weeks	10	25	-	7.68	5.68	2.00
		33	-	7.00	7.40	2.00
3 Weeks	11	25	6.77	7.70	5.75	<2.00
		33	7.92	5.97	6.05	<2.00
3 Weeks	12	25	-	7.68	6.18	<2.00
		33	-	5.98	6.84	<2.00

* Not investigated
▪ Analysed in treatment 2, 3, 5, 7, 9, 11
• Analysed in treatment 2, 4-12
▲ Analysed in treatment 5-12

Table 11 pH analyses and amount of organic acid forming during preservation of the inoculated wheat. Data represents mean value (n=2).

Preservation time	Treatment	Moisture content (%)	pH	Concentration (mg/g dry matter)				
				Acetic acid	Lactic acid	Propionic acid	Formic acid	Succinic acid
0 h	1-12	25	6.2	-*	-	-	-	-
		33	6.3	-	-	-	-	-
3 week	1	25	5.9	0.99	-	-	-	-
		33	6.1	-	0.64	-	0.97	-
	2	25	5.5	1.23	0.67	-	-	-
		33	4.0	3.28	18.7	0.60	-	1.05
	3	25	5.6	-	1.01	-	1.02	-
		33	4.0	1.60	17.5	0.75	-	0.90
	4	25	5.6	1.27	-	-	-	-
		33	5.7	1.49	-	0.30	1.75	0.64
	5	25	5.5	1.20	0.57	-	-	-
		34	4.0	4.24	15.2	2.69	-	0.96
	6	25	5.6	1.31	-	-	-	-
		33	5.4	1.75	-	1.82	0.90	-
	7	25	5.2	1.13	-	-	-	-
		33	4.0	3.64	17.8	-	-	1.12
	8	25	5.7	0.88	-	-	-	-
		33	5.6	1.46	1.02	-	-	0.94
	9	25	5.5	1.03	0.27	-	-	-
		33	3.9	2.70	14.7	1.16	-	1.09
	10	25	5.7	1.07	-	-	-	-
		33	5.5	2.45	0.31	1.10	0.96	1.46
	11	25	5.5	1.67	0.27	-	-	-
		33	4.0	3.58	14.0	2.70	-	0.93
	12	25	5.7	1.25	-	-	-	-
		33	4.9	3.06	-	3.19	1.08	-

* No acid detected

5. Conclusions and future research

The mould growth in the mini silos seemed to mainly be affected by *P. anomala* J121. In treatments where *P. anomala* J121 was added, inhibition of *P. roqueforti* J5 was observed. *P. anomala* J121 alone might not guarantee a safe preservation, due to their lower ability to grow in more moist wheat. Therefore, a starter culture with a combination of several organisms is to prefer. The addition of a homofermentative LAB in the starter culture can, especially in more moist wheat, caused a rapid decrease in pH. Adding PAB can enhance the stability of the preservation due to their production of propionic acid and acetic acid. Of the PAB species investigated in this study, *P. thoenii* MP11 could be used as a biocontrol agent, since it had the best ability to survive and produce acids in the silos. By prolonging the aerobic phase, the production of acids by PAB and growth of *P. anomala* J121 could be favoured. Therefore, it would be interesting to repeat the experiment with *P. anomala*, *P. thoenii* MP11, *L. plantarum* MiLAB393 and *P. roqueforti* J5 using initial aerobic phases of different length and compare the production of organic acids as well as inhibitory activity.

Using a starter culture with *L. plantarum* MiLAB393 alone will not have the ability to inhibit growth of *P. roqueforti* J5 sufficiently. This could be due to their large production of lactic acid and lack of acetic acid production. Therefore, a further study where combinations of one homo- and one heterofermentative LAB plus *P. anomala* J121 should be conducted. Most likely, the acetic acid production by heterofermentative LAB would not cause a decrease in pH low enough for the acid to inhibit growth of *P. anomala* J121 and spoilage organisms. However, when combined with homofermentative LAB, production of lactic acid would ensure that a pH low enough is obtained, resulting in a synergetic effect.

6. Acknowledgements

First of all I would like to thank Karin Jacobson and Matilda Olstorpe for giving me the opportunity to end my studies with an interesting and instructive study. Special thanks to my supervisor, Karin Jacobsson, who has supported me a lot during this thesis work and also spent weekends at SLU to enable my laboration work. I would also like to thank Karin Neil Persson and Ulrich Thieme, who gave me a lot of inspiration and many enjoyable moments during this autumn. Finally, thanks to my family and friends, who have supported me during this work.

7. References

Aidoo, K.E. (1993) Post-harvest storage and preservation of tropical crops. *International Biodeterioration and Biodegradation* 32, 161-173

Björnberg, A. & Schnürer, J. (1993) Inhibition of the growth of grain-storage molds in vitro by the yeast *Pichia anomala* (Hansen) Kurtzman. *Canadian Journal of Microbiology* 36, 623-628

Broberg, A., Jacobsson, K., Ström, K. & Schnürer, J. (2007) Metabolite Profiles of Lactic Acid Bacteria in Grass Silage. *Applied and Environmental Microbiology* 73, 5547-5552

Druvefors, U., Jonsson, N., Boysen, M.E. & Schnürer, J. (2002) Efficacy of the biocontrol yeast *Pichia anomala* during long-term storage of moist feed grain under different oxygen and carbon dioxide regimens. *FEMS Yeast Research* 2, 389-394

Druvefors, U. Ä. (2004) Yeast biocontrol of grain spoilage moulds- Mode of action of *Pichia anomala*. Doctoral thesis, Swedish University of Agricultural Sciences, Uppsala

Druvefors, U.Ä., Passoth, V. & Schnürer, J. (2005) Nutrient effects on biocontrol of *Penicillium roqueforti* by *Pichia anomala* J121 during airtight storage of wheat. *Applied and Environmental Microbiology* 71, 1865-1869

Druvefors, U. Ä. & Schnürer, J. (2005) Mold-inhibitory activity of different yeast species during airtight storage of wheat grain. *FEMS Yeast Pesearch* 5, 373-378

Grinstead, D. A. & Barefoot, S. F. (1992) Jenseniin G, a heat-stable bacteriocin produced by *Propionibacterium jenseii* P126. *Applied and Environmental Microbiology* 58, 215-220

Hocking, A.D. (2003) Microbiological facts and fictions in grain storage. In: Proceedings of the Australian Postharvest Technical Conference. Wright EJ, Webb MC, Highley E, eds. Canberra: CSIRO: 55-58

Holzer, M., Mayrhuber, E., Danner, H. & Braun, R. (2003) The role of *Lactobacillus buchneri* in forage preservation. *TRENDS in Biotechnology* 21, 282-287

Klijn, N., Nieuwenhof, F.F.J., Hoolwerf, J.D., van der Waals, C. B. & Weerkamp, A.H. (1995) Identification of clostridium as the causative agent of late blowing in cheese by species-specific PCR amplification. *Applied and Environmental Microbiology* 61, 2919-2924

Lambert, R.J. & Stratford, M. (1999) Weak-acid preservatives: modelling microbial inhibition and response. *Journal of Applied Microbiology* 86, 157-164

Lind, H., Jonsson, H. & Schnürer, J. (2005) Antifungal effect of diary propionibacteria-contribution of organic acids. *International Journal of Food Microbiology* 98, 157-165

Lindgren, S. (2008) Microbes for food fermentations: Risks and benefits (lecture). In: *Microbes and the law- setting the limits for practical use*, Uppsala, 2008-10-07

Madigan, M. T. & Martinko, J. M. (2006) *Biology of microorganisms*. 11 ed. United States of America: Pearson Prentice Hall

Magnusson, J. & Schnürer, J. (2001) *Lactobacillus coryniformis* subsp. *coryniformis* Strain Si3 Produces a Broad-Spectrum Proteinaceous Antifungal Compound. *Applied end Environmental Microbiology* 67, 1-5

Middelhoven, W. J. & van Balen, A. H. M. (1988) Development of the yeast flora of whole-crop maize during ensilling and during subsequent aerobiosis. *Journal of Animal Feed Sciences* 42, 199-207

Merry, R. J. & Davies, D. R. (1999) Propionibacteria and their role in the biological control of aerobic spoilage in silage. *Lait* 79, 149-164

Olstorpe, M. (2008) Feed grain improvement through biopreservation and bioprocessing. Doctoral thesis, Swedish University of Agricultural Sciences, Uppsala

Olstorpe, M., Schnürer, J. & Passoth, V. (2008) Microbial changes during storage of moist crimped cereal grain under Swedish farm conditions. (Submitted)

Petersson, S. & Schnürer, J. (1995) Biocontrol of mould growth in high-moisture wheat stored under airtight conditions by *Pichia anomala, Pichia guilliermondii and Saccharomyces cerevisiae*. *Applied and Environmental Microbiology* 61, 1027-1032

Petersson, S. (1998) *Yeast/Mold interaction during airtight storage of high-moisture feed grain*. Doctoral thesis, Swedish University of Agricultural Sciences, Uppsala

Petersson, S., Jonsson, N. & Schnürer, J. (1999) *Pichia anomala* as a biocontrol agent during storage of high-moisture feed grain under airtight conditions. *Postharvest Biology and Technology* 15, 175-184

SCB, El- och gaspriser jan1997(2001)-jan 2007, http://www.scb.se/templates/Product____24716.asp, (2008-12-12)

Schnürer, J. & Magnusson, J. (2005) Antifungal lactic acid bacteria as biopreservatives. *Trends in Food Science and Technology* 16, 70-78

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

SJV (2008) *Yearbook of agricultural Statistics 2008*. Swedish Broad of Agriculture. Stockholm

SJVFS 2006:81, http://www.sjv.se/download/18.1c0adca10f809866b68000479/Allm%C3%A4nna+r%C3%A5d+om+foderhygien.pdf, (2008-12-09)

Ström, K. (2005) Fungal inhibitory lactic acid bacteria- Characterization and application of *Lactobacillus plantarum* MiLAB 393. Doctoral thesis, Swedish University of Agricultural Sciences, Uppsala

Ström, K., Schnürer, J. & Melin, P. (2005) Co-cultivation of antifungal *Lactobacillus plantarum* MiLAB393 and *Aspergillus nidulans*, evaluation of effects on fungal growth and protein expression. *FEMS Microbiology Letters* 246, 119–124

Thougaard, H., Varlund, V. & Madsen, R. M. (2001) *Grundläggande Mikrobiologi*. Lund: Studentlitteratur

Weinberg, Z.G. & Muck, R.E. (1996) New trends and opportunities in the development and use of inoculants for silage. *FEMS Microbiology Reviews* 19, 53-68

Appendix 1

Growth media

GPY-glucose peptone yeast extract

0.04 %	Glucose
1.00 %	Bacteriological Peptone
0.05 %	Yeast extract

MEA-soft agar

1.00 %	Agar technical (Agar NO.3)
0.05 %	Malt extract

NL-agar (Grinstead and Barefoot, 1992)

1.00 %	Sodium lactate
1.00 %	Yeast extract
1.00 %	Trypticase soy broth without dextrose
1.20 %	Agar technical (Agar NO.3)

NL-broth

1.00 %	Sodium lactate
1.00 %	Yeast extract
1.00 %	Trypticase soy broth without dextrose

Peptone water

0.20 %	Bacteriological Peptone
0.01 %	Tween 80

Appendix 2

Ympning av mikroorganisimer i spannmål kan minska tillväxt av oönskade organismer under lagerhållningen

I tempererade länder som Sverige, där mycket regn faller under skördeperioden, kan höga vattenhalter i spannmål orsaka problem vid lagerhållningen. Tillväxt av spannmålens naturliga mikroflora gynnas av en hög vattenhalt, vilket kan resultera i stor oönskad tillväxt av till exempel mögelsvampar. Den oönskade tillväxten förbrukar näring, vilket leder till att spannmålets näringsvärde sänks. Mögelsvamparna har förutom detta även förmågan att producera olika gifter, mykotoxiner. För att minska tillväxten av oönskade organismer krävs en effektiv och säker lagringsmetod.

Idag torkas spannmålen före lagring för att sänka vattenhalten och på så sätt förhindra oönskad tillväxt av skadliga organsimer. Torkning är en effektiv men energikrävande process. En alternativ metod är lufttät lagring, vilket kräver betydligt mindre energi, då principen bygger på att frånvaro av syre och förhöjda halter av koldioxid hämmar skadliga organsimer. Den syrefria miljön uppstår tack vare cellandningen hos spannmålens mikroflora.

Genom att använda biokonservering, där man tillsätter en startkultur med en eller flera organismer, kan lagerhållningen förbättras och kvalitén på den lagrade spannmålen höjas. Organismerna i startkulturen modifierar miljön i systemet genom att till exempel förbruka syre, vilket leder till att den syrefria miljön uppnås snabbare. De konkurrerar också om utrymme och näring och kan även producera hämmande föreningar. För att garantera en säker lagerhållning, oavsett spannmålens fukthalt, kan en startkultur av flera organismer vara att föredra, eftersom organismerna är verksamma vid olika vattenhalter. I ett examensarbetet, utfört på SLU, Uppsala, har olika organismers förmåga att inhibera möglesvampen *Penicillium roqueforti* i lagrad spannmål med en fukthalt på 24-33 % studerats. Organismer som studerades var följande; jästen *Pichia anomala* J121, mjölksyrabakterien *Lactobacillus plantarum* MiLAB393(pLV100) samt fyra olika propionsyrabakterier (PAB).

Av de organismer som undersöktes, konstaterades att jästen *Pichia anomala* J121 hade störst inverkan på mögeltilväxten. Trots att jästen uppvisade viss tendens på att växa bättre vid lägre fukthalter, hade den god förmåga att inhibera mögel även i fuktigare spannmål.

Generellt sågs en klar skillnad i pH reduceringen mellan fukthalterna. Den kraftigaste minskningen observerades i det fuktigare spannmålen. En god tillväxt av den homofermentativa *Lactobacillus plantarum* MiLAB393(pLV100), vilken producerar stor mängd mjölksyra, i fuktigare spannmål förklarar pH skillnaden mellan fukthalterna. Enligt undersökningarna har dock inte *Lactobacillus plantarum* MiLAB393(pLV100) själv förmågan att inhibera mögel vid de aktuella fukthalterna. Trots ett lågt pH sågs en tydlig mögeltillväxt i spannmål, där startkultur med enbart *Lactobacillus plantarum* MiLAB393(pLV100) hade använts. Troligtvis var inte pH tillräckligt lågt för att få mjölksyran i dess aktiva, oladdade form. Den aktiva formen kan passera över cellers membran och påverkar tillväxtfasen. Trots att studien visade att enbart en hög

mjölksyreprodutkionen inte har förmågan att hämma mögeltillväxt, kan en startkultur med en homofermentativ mjölksyrabakterie föredras. Dess bidrag till pH reduceringen leder till att propionsyra och ättiksyran som produceras av jästen och PAB kan vara i dess aktiva form och har därmed påverka tillväxtfasen hos andra celler.

PAB hade generellt svårt att växa och överleva i spanmålen. De tycktes dock föredra fuktigare miljöer. Där kunde en tillväxt samt produktion av propionsyra detekteras. PAB påverkades negativt av en stor tillväxt av *Lactobacillus plantarum* MiLAB393(pLV100). Större mängd PAB kunde observeras då startkulturer utan *Lactobacillus plantarum* MiLAB393(pLV100) användes jämfört med startkulturer där mjölksyrabakterien ingick. PAB har i tidigare studier visat sig känslig mot en snabb pH sänkning orsakat av mjölksyrabakteriers mjölsyraproduktion.

Tack vare jästens lägre förmåga att växa i fuktigare spannmål kan en tillsatts av en LAB och en PAB i stratkulturen föredras. I studien visade bakterierna på god förmåga att växa och producera syror vid höga fukthalter. Tidigare studier påvisar att PAB produktion kan gynnas av en förlängd inledande syrefas under lagringen, vilket fördröjer en snabb pH sänkning orsakat av LAB. I samband med att koncentrationen av PAB ökar kan mer syra produceras och därmed kan kvalitén på den lagrade spannmålen ökas. Med tanken på detta är det intressant att genomföra nya undersökningar, där den inledande fasen med syre närvarande varieras i längd.

Det skulle även vara intressant att studera en startkultur med *P. anomala* J121, en homo- och en heterofermentativ LAB. Den heterofermentativa producerar mjölksyra, dock mindre mängd än vad den homofermentativa producerar, samt ättiksyra. På grund av dess låga mjölksyraproduktion kan de inte själva bidra med en kraftig reducering av pH, vilket krävs för att ättiksyran ska vara i dess aktiva form. En kombination av dessa två LAB skulle dock kunna resultera i en synergistisk effekt där den heterofermetativa sänker pH, vilket förstärker ättiksyrans inhiberingsförmåga.