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Arja Laitila

Microbes in the tailoring of barley malt properties

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Arja Laitila

Academic dissertation in Microbiology

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Cover picture: Microbial ecosystem in malting under the spotlight. FESEM micrograph of *Lactobacillus plantarum* VTT E-78076 cells adhered to barley seed-coat tissues, Mari Raulio, University of Helsinki & Arja Laitila, VTT. Visual editing Arja Laitila & Kaarina Takkunen, VTT.

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Keywords barley, malting, malt quality, bacteria, yeasts, filamentous fungi, microbiota, management, biocontrol

Abstract

Malted barley (malt) is traditionally used in the production of beer and distilled spirits. In addition, it can be processed into ingredients for different areas of the food industry. Malting, the controlled germination of cereal grains, is a complex biological process involving a wide range of biochemical and physiological reactions. The diverse microbial communities naturally colonizing barley grains play a crucial role in this process. Therefore, the malting process can be considered as an ecosystem involving two metabolically active groups: the germinating grains and the diverse microbiota. It is evident that the multitude of microbes greatly influences the malting process as well as the quality of the final product. The main goal of this thesis was to study the relationships between microbes and the germinating grain during the malting process. Furthemore, this study provides a basis for tailoring of malt properties with natural, malt-derived microbes.

The results of this study showed that the malting ecosystem is indeed a dynamic process and exhibits continuous change. Microbes embedded in biofilms within the husk tissues were well protected. Reduction of one population within the complex ecosystem led to an increase in competing microbes. This should be taken into account when changes are made in the malting process. Using different molecular approaches we also found that the diversity of microbes in malting was much greater than previously anticipated. Some potentially novel bacterial and fungal species were found in the malting ecosystem.

The microbial communities greatly influenced grain germination and malt properties. By suppressing Gram-negative bacteria during steeping, barley vitality and malt brewhouse performance were improved even in the case of good-quality malting barley. The fungal community consisting of both yeasts

and filamentous fungi significantly contributed to the production of microbial β-glucanases and xylanases, and was also involved in the proteolysis.

Previously the significance of yeasts in the malting ecosystem has been largely underestimated. This study showed that a numerous and diverse yeast community consisting of both ascomycetous (25) and basidiomycetous (18) species occured in the industrial malting ecosystem. Yeast and yeast-like fungi produced extracellular hydrolytic enzymes with a potentially positive contribution to malt processability. Furthermore, several yeast strains showed strong antagonistic activity against field and storage moulds.

The management of microbes in the whole barley-malt-beer chain is extremely important with respect to both process and product safety and quality. Lactic acid bacteria (LAB) can be used to tailor the malt properties. *Lactobacillus plantarum* VTT E-78076 (E76) and *Pediococcus pentosaceus* VTT E-90390 (E390) added to steeping water promoted yeast growth and restricted the growth of Gram-negative bacteria and *Fusarium* fungi. Furthermore, they had positive effects on malt characteristics and notably improved wort separation. Some of the beneficial effects observed with LAB were due to the lactic acid production and concomitant lowering of pH. Futhermore, increase in the number of yeasts could partly explain the enhanced xylanase and β-glucanase levels observed after LAB addition.

Addition of a specific yeast culture (*Pichia anomala* VTT C-04565) into the steeping water of barley restricted *Fusarium* growth and hydrophobin production during malting and thus prevented beer gushing. This study also revealed that *P. anomala* retarded the wort filtration, but that the filtration performance was recovered when yeast cultures were combined with *L. plantarum* E76. The combination of different microbial cultures offers a possibility to utilise their different properties, thus making the system more robust. Improved understanding of the complex microbial communities in the malting ecosystem will enable more efficient control of unwanted microbiological phenomena as well as utilization of the beneficial properties of microbes in malt production.

Laitila, Arja. Microbes in the tailoring of barley malt properties [Mikrobit ohramaltaan ominaisuuksien muokkaajana]. Espoo 2007. VTT Publications 645. 107 s. + liitt. 79 s.

Avainsanat barley, malting, malt quality, bacteria, yeasts, filamentous fungi, microbiota, management, biocontrol

Tiivistelmä

Mallastusprosessi voidaan määritellä ekosysteemiksi, joka koostuu itävästä jyvästä ja jyvän kanssa läheisesti elävästä monimuotoisesta mikrobiyhteisöstä. Mallastuksen mikrobiyhteisön monitorointi, ohjaus ja hallinta ovat ensiarvoisen tärkeässä asemassa, koska mikrobit vaikuttavat oleellisesti maltaan prosessiteknisiin ominaisuuksiin, mikrobiologiseen turvallisuuteen sekä lopputuotteen laatuun. Tässä tutkimuksessa perehdyttiin bakteerien, hiivojen ja homeiden vaikutuksiin ohran itämisen ja maltaan laadun kannalta. Bakteeri- ja sieniyhteisön, erityisesti hiivojen, tunnistamisessa hyödynnettiin perinteisten mikrobiologisten määritysmenetelmien lisäksi uusia molekyylibiologisia tunnistusmenetelmiä. Keskeinen tutkimuskohde oli mallastusprosessiin soveltuvien mikrobiyhteisön hallintakeinojen kartoittaminen. Tutkittiin erityisesti mallastuksen luontaisten maitohappobakteerien ja hiivojen hyötykäyttöä ekosysteemin ohjauksessa.

Tutkimus osoitti, että mikrobeilla oli keskeinen rooli mallastuksessa. Mallastusprosessissa vallitsi mikrobien kasvun kannalta edulliset olosuhteet, ja ohramatriisissa esiintyvä monimuotoinen mikrobiyhteisö pystyi mukautumaan erittäin nopeasti vaihtuviin ympäristöolosuhteisiin. Ohran liotuksen ja maltaan kuivauksen alkutunnit olivat mikrobiologisesti kriittiset pisteet. Mikrobiyhteisö osoittautui huomattavasti monipuolisemmaksi kuin aiemmin oli osoitettu. Mallastuksesta tunnistettiin uusia bakteeri- ja hiivalajeja. Mikrobiyhteisöä muokkaamalla voitiin parantaa ohran itämistä ja maltaan prosessiteknisiä ominaisuuksia. *Lactobacillus plantarum* VTT E-78076- ja *Pediococcus pentosaceus* VTT E-90390 -maitohappobakteerien lisäys ohran liotusveteen rajoitti maltaan prosessointia haittaavien bakteerien ja homeiden kasvua.

Tuotantomallastusten hiivayhteisön perusteellinen kartoitus osoitti, että tästä ryhmästä löytyi runsaasti hyödyllistä entsyymipotentiaalia. Lisäksi tiettyjen hiivojen

avulla estettiin haittahomeiden, erityisesti *Fusarium*-sienten, kasvua. Mallastuksen luontaiseen mikrobiyhteisöön kuuluvan *Pichia anomala* VTT C-04565 -hiivan lisäys ohran liotusveteen esti *Fusarium*-sienten tuottamien oluen ylikuohunta-tekijöiden muodostumisen mallastuksessa. Toisaalta tämä hiiva yksinään lisättynä hidasti vierteen erotusta. Epäedulliset vaikutukset maltaan prosessointiin voitiin kuitenkin poistaa, kun *P. anomala* C565 -hiivaa käytettiin yhdessä *L. plantarum* E76 -maitohappobakteerin kanssa.

Uusien monitorointi- ja ohjauskeinojen avulla on mahdollista päästä nykyistä paremmin hallittuun ja ennakoivaan prosessiin, jossa voidaan täsmällisemmin räätälöidä mallastettujen viljojen laatuparametrejä lopputuotteiden käyttäjien tarpeiden mukaisesti.

Academic dissertation

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This book is dedicated

in loving memoriam

to my father Kauko Laitila

Preface

This study was carried out at VTT Technical Research Centre of Finland during the years 2001–2007. The research was part of the “Barley to Beer Quality” research programme conducted by the Finnish Malting and Brewing Research Laboratory (Oy Panimolaboratorio Ab). The research was funded by the Finnish Malting and Brewing Industry and Tekes – the Finnish Funding Agency for Technology and Innovation. Furthermore, financial support for this thesis work was obtained from VTT, from the Emil Aaltonen Foundation, and from the Raisio Group Research Foundation. The support of all these companies, foundations and organizations is gratefully acknowledged.

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My dearest thanks are due to my family, to my relatives, to my lovely godchildren and to my numerous friends, who have shared all the joys and sorrows in everyday life. You are my life! What a wonderful journey we are on. Special big thanks are due to our girlgang Anne, Anu, Liisa, Meri, Päivi, Riikka and Ritu. I would like to say thank you from the bottom of my heart for your true friendship. I am also looking forward to new adventures.

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List of original publications

This thesis is based on the following original publications, referred to in the text by their Roman numerals (Papers I–IV). In addition, some unpublished data are presented.

I Laitila, A., Kotaviita, E., Peltola, P., Home, S. and Wilhelmson, A. 2007. Indigenous microbial community of barley greatly influences grain germination and malt quality. Journal of Institute of Brewing 113:9–20.

II Laitila, A., Sweins, H., Vilpola, A., Kotaviita, E., Olkku, J., Home, S. and Haikara, A. 2006. *Lactobacillus plantarum* and *Pediococcus pentosaceus* starter cultures as a tool for microflora management in malting and for enhancement of malt processability. Journal of Agricultural and Food Chemistry 54:3840–3851.

III Laitila, A., Wilhelmson, A., Kotaviita, E., Olkku, J., Home, S. and Juvonen, R. 2006. Yeasts in an industrial malting ecosystem. Journal of Industrial Microbiology and Biotechnology 33:953–966.

IV Laitila, A., Sarlin, T., Kotaviita, E., Huttunen, T., Home, S. and Wilhelmson, A. 2007. Yeasts isolated from industrial maltings can suppress *Fusarium* growth and formation of gushing factors. Journal of Industrial Microbiology and Biotechnology, submitted, revised.

Papers I–IV are reprinted with permission from the respective publishers.

Author's contribution

I Arja Laitila is the corresponding author. She was responsible for microbiological analyses and PCR-DGGE. Planning of the research, experimental design, interpretation of the results and writing the paper was carried out together with Annika Wilhelmson, who was also responsible for monitoring the grain germination and physiology.

II Arja Laitila had the main responsibility for preparing and writing the article and is the corresponding author. She planned the study, was responsible for the experimental work and interpreted the results, except that the evaluation of malt physical and chemical properties was performed together with Hannele Sweins and Silja Home.

III Arja Laitila had the main responsibility for preparing and writing the article and is the corresponding author. She planned the study, was responsible for experimental work and interpreted the results, except that the sequence analysis of the 26S rRNA gene was performed together with Riikka Juvonen.

IV Arja Laitila had the main responsibility for preparing and writing the article and is the corresponding author. She planned the study, was responsible for experimental work and interpreted the results, except that the hydrophobin analysis was carried out by Tuija Sarlin and the evaluation of malt physical and chemical properties was performed together with Annika Wilhelmson.

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List of symbols

a_w	Water activity, a measure of free water in foods
ADH	Alcohol dehydrogenase
BA	Biological acidification
CFU	Colony forming unit
CMC	Carboxymethyl cellulose
CZID	Czapek-Dox agar, containing Iprodion and Dichloral
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
EBC	European Brewery Convention
EPS	Exopolysaccharide
FAN	Free Amino Nitrogen
FESEM	Field Emission Scanning Electron Microscopy
FHB	*Fusarium* Head Blight
FISH	Fluorescence In Situ Hybridization
HACCP	Hazard Analysis and Critical Control Points
IFBM	French Institute of Brewing and Malting
kGy	Kilogray, an SI unit used to measure the absorbed dose of radiation
LAB	Lactic acid bacteria
MFI	Mold Frequency Index
MRS	de Man-Rogosa-Sharpe medium for lactic acid bacteria
MRS-LA	MRS medium supplemented with Lactic Acid
PCR-DGGE	Polymerase Chain Reaction – Denaturing Gradient Gel Electrophoresis
PYF	Premature Yeast Flocculation
RNA	Ribonucleic Acid
RT-PCR	Reverse transcription (RT) PCR
TBE	Tris-Borate-EDTA buffer
TRAC	Transcript Analysis with the aid of Affinity Capture
VTT	VTT Technical Reasearch Centre of Finland
YM	Yeast-Malt Extract medium

1. Introduction

Beer is one of the oldest beverages known to man. It is also one of the most widespread drinks, found on every continent and in every culture worldwide. The major proportion of the world's beers is produced from malted barley. Approximately 17 million tons of barley malt was produced in 2004, of which 43% was produced in EU countries (Rabobank International, World Beer and Malt Map 2004). Malt is also used in the production of distilled spirits, and it can be processed into ingredients for different branches of the food industry (Pyler & Thomas 2000). Recently, non-alcoholic, malt-based beverages with a healthy image have gained considerable interest. In addition to barley (*Hordeum vulgare* L, Poaceae), many other cereals such as oat and wheat are malted (Davies 2006, Kaukovirta-Norja *et al.* 2004). Malt provides nutrients for yeast growth, such as fermentable sugars and low molecular weight nitrogenous compounds needed in beer fermentation processes. Moreover, malt has a great effect on the brewing performance and on the characteristics of the final beer (Bamforth 2001). In addition, malting generally improves the nutritional value of cereals by enhancing the production of valuable bioactive compounds such as vitamins.

1.1 Malting ecosystem

The production of malt (malting) is a complex biological process involving a wide range of biochemical and physiological reactions (Bamforth & Barclay 1993). The main goal is to produce various enzymes capable of degrading the grain macromolecules into soluble compounds. This enzyme-catalyzed breakdown of the grain endosperm structure is called malt modification. The outward appearance of the final malt resembles that of the unmalted barley, but the physical, biochemical and microbiological composition is changed.

Malting traditionally involves three stages: steeping, germination and kilning. Figure 1 shows a simplified scheme of the malt and beer production process. During steeping, the moisture content of the grains is increased at 13–20 °C up to 43–46% by alternating immersion and air rest periods. The steeping water is generally aerated. Furthermore, air rests are introduced into the steeping process

to improve oxygen availability, as barley is not an aquatic plant and can be damaged if immersed in water for prolonged periods. The grains are then allowed to germinate under humid and aerobic conditions at 16–20 °C for 3–6 days. During germination, temperated aeration through the grain bed is used to control the germination temperature. In addition, the grain bed is turned regularly to avoid temperature gradients and matting of barley rootlets. Aeration also plays an important role in removing carbon dioxide that can have a negative impact on grain germination. Finally, germination is terminated by kilning (drying) the grains for approximately 21 h at temperatures increasing gradually from about 50° to 85 °C or more depending on the type of malt. Kilning halts the biochemical reactions and ensures microbiological stability of the dried product (final moisture content 3–4%). Furthermore, several colour and flavour compounds are produced during kilning.

In the brewery, malt is milled and mashed with water. In the mashing stage, malt enzymes break down the grain components into fermentable sugars and other yeast nutrients. The watery mixture with dissolved substances, wort, is separated from the grain insoluble parts (spent grains) during lautering. Barley husks act as a filter material in wort separation. Two main technologies are employed at this stage, namely lauter tun and the mash filter. After boiling with hops and cooling, wort is ready for beer fermentation (Figure 1).

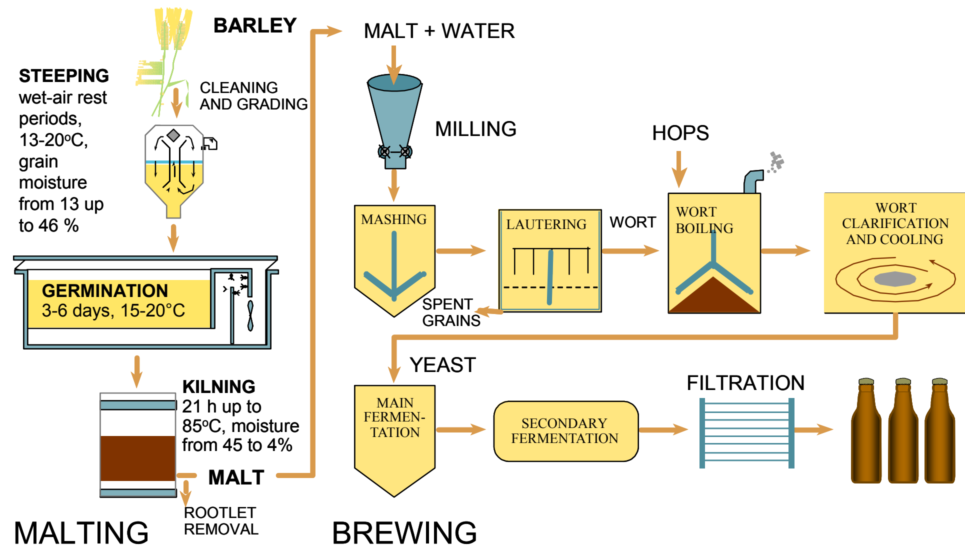

Figure 1. Malting and brewing processes.

Malting utilizes the natural physiological process, grain germination, during which the considerable biochemical potential of the grain is utilized. In addition to germinating grain, the malting process includes another metabolically active component: a diverse microbial community that includes various types of bacteria, yeasts and filamentous fungi (discussed in detail in Section 1.2). Therefore, malting can be considered as a complex ecosystem involving two metabolically active groups: the barley grains and the diverse microbial community (Figure 2). It is evident that the multitude of microbes has a significant impact on malting and brewing performance as well as on the quality of malt and beer.

The grain ecosystem is greatly influenced by the whole history experienced by the grain during the growth period, harvesting and storage. Furthermore, the behaviour of both barley and microbes during the malting process is influenced by multiple interactive factors such as moisture, temperature, gaseous atmosphere and time. Whenever the malting process is changed, both grain and microbial activity should be considered.

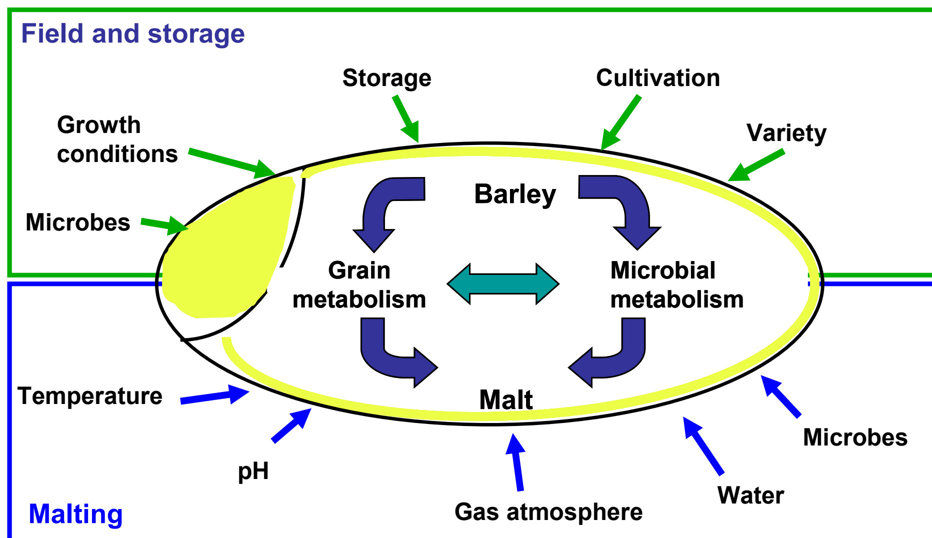

Figure 2. Factors influencing the malting ecosystem.

1.2 Microbial ecology of barley and malting

1.2.1 Microbial community in barley

The microbial community characteristic to malted barley products develops in the field, under storage, and during the processing (Figure 3). Many intrinsic and extrinsic factors including plant variety, climate, soil type, agricultural practices, storage and transport influence the diversity and structure of the microbial community present in the barley grains (Angelino & Bol 1990, Douglas & Flannigan 1988, Flannigan 2003, Haikara *et al.* 1977, Petters *et al.* 1988). Of these climate is believed to play a particularly important role (Etchevers *et al.* 1977). Therefore, barleys cultivated in different geographic locations have different microbial communities. The composition of the microbial community on barley grains changes dramatically as a result of post-harvest operations (Figure 3). Some of the grain-associated microbes are removed during processing of grains, whereas every process step in the barley-malt-beer chain can be a source of additional microbial populations. A stored barley batch as well as the grain bed in malting can be considered as a man-made ecosystem, in which the live barley tissues can interact with the surrounding environment and microbes.

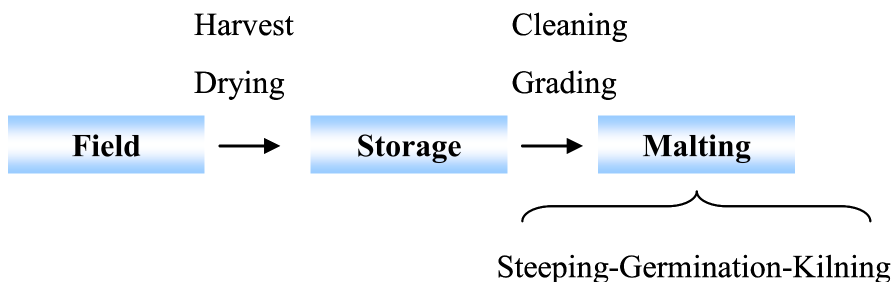

Figure 3. Three ecological niches for microbial communities in malting barley.

Barley grain is composed of three major parts: the embryo, the endosperm and a protective layer including the husk, the pericarp and the testa, also known as the seed coat (Figure 4). The husk mediates uniform water uptake and provides mechanical protection for the barley embryo and the primary leaf developing during the germination (the acrospire). The several different layers found in the grain coverings act as a carrier for microbes (Olkku *et al.* 2005). In the field,

barley kernels are already colonized by microbes soon after ear emergence from the enveloping leaf-sheaths. Wind, rain, insects, birds and agricultural practices effectively distribute microbes throughout the growing season (Flannigan 2003). At later stages of kernel filling, microbial colonization is restricted to the outer parts of the developing kernels, between the testa and the outer epidermis. In healthy grains, testa restricts microbial attack into the grain interior (Figure 4C). Occasionally, invasion of the endosperm is caused by fungi with distinct phytopathogenic characteristics, such as *Fusarium* fungi, or if the testa is for some reason injured (Schmidt 1991).

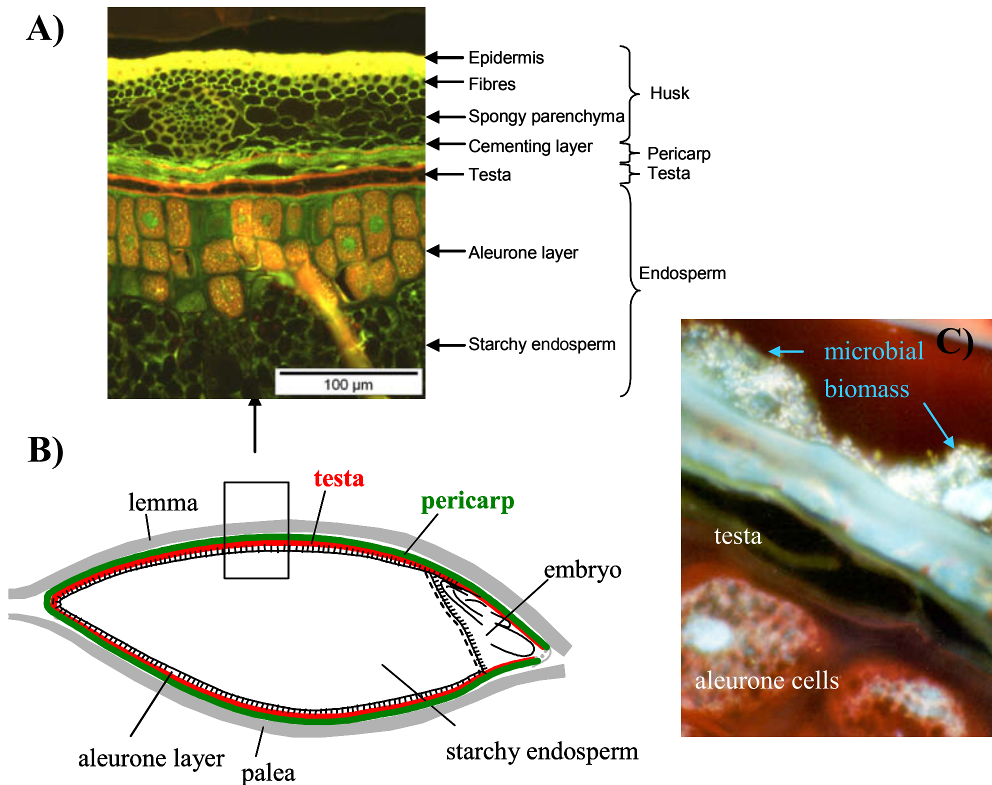

Figure 4. A) Barley grain structure. B) Structure of the outer layers in mature barley grain (reference Olkku et al. 2005). C) Microbial biomass located outside the testa layer.

Barley kernels represent a complex, non-uniform substrate for microbes with respect to physical and chemical parameters (Noots *et al.* 2003). Barley has the following average chemical composition: total carbohydrate 70–85% (including

starch, cellulose, β-glucans, pentosans and gums), protein 10.5–11%, inorganic matter 2–4%, fat 1.5–2.0% and other substances 1–2% (including polyphenols, vitamins) (Kunze 1999, Palmer 1989). The majority of the nutritional components are accumulated in the endosperm cells. The outer layers of grains, in which the significant part of the microbial community is located, consist mainly of cellulose, hemicellulose and lignin and also contain small amounts of proteins (Olkku *et al*. 2005).

It has been suggested that microbial populations adhered to external and internal surfaces of barley tissues form a compact biofilm (Thomas & Usher 2001). This multicellular mode of growth predominates in nature and provides adaptive strategies for plant-associated microbes in changing or stressful environments (Morris & Monier 2003). In a nutrient-poor environment such as on the surfaces of plant tissues, microbial cells often become filamentous to maximize their absorbing surface (Morris & Monier 2003). Biofilm-grown cells are also well protected and have shown increased resistance to external factors such as desiccation, heat and antimicrobial treatments (Costerton *et al.* 1987). However, little is known about the complex associations of microbes within grain biofilms during barley processing.

The indigenous microbial community of barley harbours a wide range of microorganisms including numerous species of Gram-negative and -positive bacteria, yeasts and filamentous fungi (Flannigan 2003, Haikara *et al.* 1977, Noots *et al.* 1999, Petters *et al.* 1988). Low levels of actinobacteria, mainly members of the *Streptomycetes* genus, occur occasionally. Table 1 shows microbes frequently detected on pre-harvest barley.

Bacteria numerically dominate the culturable microbial community of pre-harvest barley (Angelino & Bol 1990). Approximately 10 million bacteria are frequently detected in one gram of barley (Flannigan 2003, Noots *et al.* 1999). This provides an estimate that at least 500 000 bacteria can be found in a single barley kernel.

Table 1. Microbial species belonging to the listed genera are frequently detected on pre-harvest barley (Flannigan 2003, Haikara et al. 1977, Noots et al. 1999, Petters et al. 1988).

Bacteria	**Yeasts**	**Filamentous fungi**
Bacillus	*Candida*	*Alternaria*
Enterobacter	*Cryptococcus*	*Aureobasidium*
Erwinia	*Pichia*	*Cephalosporium*
Flavobacterium	*Sporobolomyces*	*Cladosporium*
Klebsiella	*Rhodotorula*	*Dreachslera*
Micrococcus	*Trichosporon*	*Fusarium*
Pseudomonas		*Epicoccum*
Streptomyces		
Xanthomonas		

Yeasts are the second most abundant culturable microbes in pre-harvest barley (Flannigan 2003). However, their numbers may be exceeded by filamentous fungi during later stages of ripening (Angelino & Bol 1990, Flannigan 2003). More than 150 species of filamentous fungi (moulds) and yeasts can be found on grains as surface contaminants or as internal invaders (Sauer *et al*. 1992). Filamentous fungi are divided into two distinct ecological groups: field and storage fungi. Among the most common and widespread field fungi in malting barley are *Alternaria, Cladosporium, Epicoccum*, *Fusarium, Cochliobolus, Drechslera* and *Pyrenophora*, the latter three formerly known as *Helminthosporium*-group (Ackermann 1998, Andersen *et al*. 1996, Flannigan 2003, Haikara *et al.* 1977, Noots *et al.* 1999). These fungi require relatively high water availability for growth ($a_w > 0.85$). Thus, their growth is restricted during storage by appropriate drying of barley.

After harvest, barley grains are stored from about two months to one year to allow the break up of the normal dormancy before malting (Pyler & Thomas 2000). Microbes are not usually active and their number generally decreases during storage under appropriate conditions (Beck *et al.* 1991, Haikara *et al.* 1977, Laitila *et al.* 2003). Microbial growth and spoilage of stored barley are determined especially by water activity and temperature (Angelino & Bol 1990). Xerophilic *Aspergillus*, *Eurotium* and *Penicillium* are the most characteristic fungi found in the storage environment (Pitt & Hocking 1997, Samson *et al.*

2000). Storage fungi are habitually present in the dust and air of the storage environment, and can also be found in different farm and malting equipments such as harvesters and elevators (Sauer *et al.* 1992). However, the differentiation into field and storage fungi is applicable only in temperate climates, since in warmer regions some species normally considered as storage fungi may be found already in the developing barley (Medina *et al.* 2006, Noots *et al.* 1999).

1.2.2 Evolution of microbial populations during malting

The microbial ecology of barley changes during malting. Before entering the malting process, barley is cleaned and graded in order to remove foreign material, dust, and small and broken kernels. Cleaning procedures also diminish the microbial load. However, malting conditions are extremely favourable for microbial growth in terms of available nutrients, temperature, moisture content and gaseous atmosphere. Figure 5 illustrates the growth of bacteria and yeasts in the industrial malting ecosystem (Wilhelmson *et al.* 2003). Steeping of barley leads to leakage of nutrients into steeping water and rapidly activates the dormant microbes present in barley grains (Kelly & Briggs 1992). Although some of the microbes and soluble nutrients are washed away along with steep water draining, the viable microbial numbers increase markedly during the steeping period (Briggs & McGuinness 1993, Douglas & Flannigan 1988, Flannigan *et al.* 1982, O'Sullivan *et al.* 1999, Petters *et al.* 1988). The steeping vessel and the water remaining at the bottom of the tank between steeps are known to serve as inocula for the next batches (O'Sullivan *et al.* 1999). Steeping is generally regarded as the most critical step in malting with respect to microbiological safety (Noots *et al.* 1999).

Microbial activity remains high throughout the germination period. Furthermore, microbial growth is accelerated during the first hours of kilning (Wilhelmson *et al.* 2003). The kilning regime has been identified as a significant factor in controlling microbial communities (Stars *et al.* 1993). Although high temperatures effectively restrict the growth and activity of microbes, kilning appears to have little effect on the viable counts of bacteria and fungi. The viable counts of microbes are generally higher in the finished malt than in native barley (Noots *et al.* 1999). Barley dries progressively from the bottom to the top of the grain bed, and the time that barley is exposed to each temperature depends on its

location in the kiln. Reduction of microbial activity depends on the moisture level and the length of time before the temperature breakthrough in the grain bed (Wilhelmson *et al.* 2003). Furthermore, the microbial community is also significantly influenced by the malthouse operations, and it has been shown that a specific microbial community develops in each malting plant (O'Sullivan *et al.* 1999, Petters *et al.* 1988). The microbial community of final malt reaching the brewery or distillery is naturally influenced by the handling and storage operations after the malting process as well as during transport of malt (Angelino & Bol 1990).

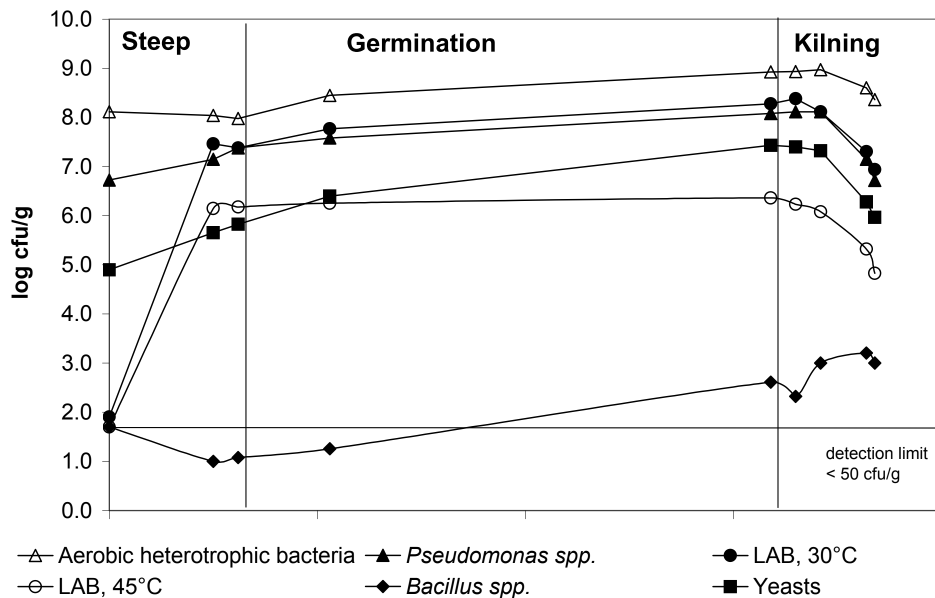

Figure 5. Growth of aerobic heterotrophic bacteria, Pseudomonas spp., mesophilic (LAB, 30 °C) and thermophilic lactic acid bacteria (LAB, 45 °C), aerobic spore-forming bacteria and yeasts during industrial scale malting. The counts are mean values obtained from different industrial malting experiments (Wilhelmson et al. 2003).

Enterobacteria and *Pseudomonas* spp. are the predominant bacteria during malting, reaching 10^8–10^9 cfu/g during germination (Douglas & Flannigan 1988, Haikara *et al.* 1977, Noots *et al.* 1999, O'Sullivan *et al.* 1999, Petters *et al.* 1988). Lactic acid bacteria (LAB) constitute only a small minority of the bacterial community in native barley. However, their numbers increase

significantly to 10^6–10^8 cfu/g during the steeping process (Booysen *et al*. 2002, Haikara *et al.* 1977, O'Sullivan *et al.* 1999, Petters *et al.* 1988, van Waesberghe 1991). Malting equipment has been shown to act as a reservoir of additional LAB (O'Sullivan *et al.* 1999). The LAB population is dominated by heterofermentative leuconostoc species during steeping, whereas lactobacilli begin to dominate during germination (Booysen *et al.* 2002, O'Sullivan *et al.* 1999, van Waesberghe 1991). However, great variation in species diversity has been observed between different malting houses.

High numbers of yeasts and yeast-like fungi have been observed during the malting process (Bol & Huis in't Veld 1988, Douglas & Flannigan 1988, Flannigan *et al.* 1982, Flannigan 2003, Haikara *et al.* 1977, O'Sullivan *et al.* 1999, Petters *et al.* 1988, Wilhelmson *et al.* 2003). Traditionally yeasts in the malting ecosystem have been roughly divided into white and pink yeasts based on the colony colour (Flannigan 2003). Previously, 10 ascomycetous and 6 basidiomycetous yeasts species were reported from barley and malting samples (Douglas & Flannigan 1988, Flannigan 1969, Flannigan & Dickie 1972, Flannigan *et al.* 1982, Flannigan 2003, Kottheimer & Christensen 1961, Noots *et al.* 1999, Petters *et al.* 1988, Tuomi *et al.* 1995, Tuomi & Rosenqvist 1995). Furthermore, a yeast-like fungus *Aureobasidium pullulans* is commonly encountered in pre- and post-harvest barley samples (Clarke & Hill 1981, Flannigan 1969, Flannigan *et al.* 1982, Hoy *et al.* 1981). However, the diversity and the role of yeasts in the malting ecosystem are still largely unknown.

The genus *Fusarium* is the most important group of filamentous fungi related to barley and malting. The species of fusaria are adapted to different ecological niches all over the world as saprophytes and plant pathogens with a wide range of host plants. Currently, over 70 species are included in this genus (Leslie & Summerell 2006). The malting environment is extremely favourable for *Fusarium* fungi (Douglas & Flannigan 1988, Haikara *et al.* 1977). As seen from Figure 6, intensive *Fusarium* growth has been observed during steeping, even when the original barley had only a low level of *Fusarium* contamination (Laitila *et al.* 2002). Approximately 30–50% higher *Fusarium* counts were measured after the steeping stage compared with the original contamination of barley. The levels of other field fungi such as *Alternaria* and *Cladosporium* usually decline during germination (Douglas & Flannigan 1988, Haikara *et al.* 1977). However, great variation in fungal communities has been observed due to

the differences in malting practices in different locations (Ackermann 1998, Douglas & Flannigan 1988, Flannigan 2003). Certain heat-resistant fungi, such as *Rhizopus* and *Mucor,* are frequently encountered at the end of germination and they continue to grow during the early hours of kilning (Douglas & Flannigan 1988, Haikara *et al.* 1977).

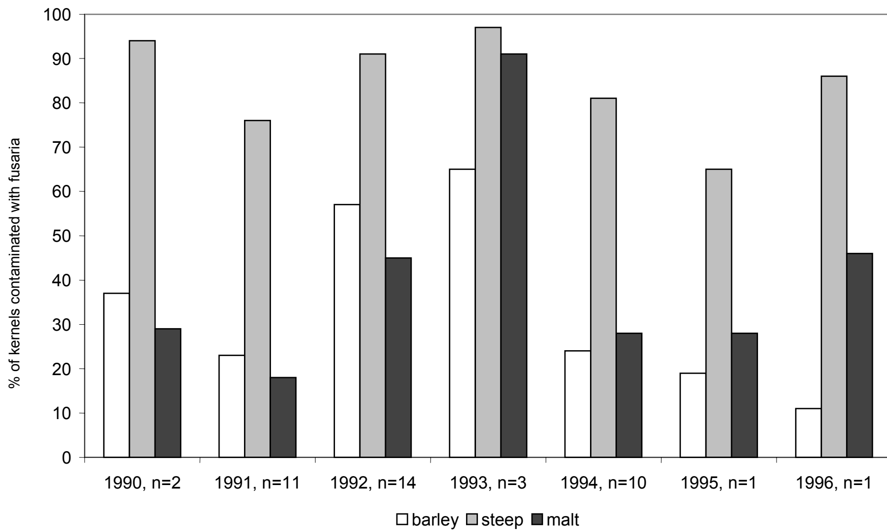

Figure 6. Fusarium fungi in laboratory scale maltings of Kymppi barley. The data were collected from malting experiments carried out in 1991–1997 (Laitila et al. 2002).

1.3 Impact of microbes on grain germination and malt quality

It is evident that the diverse microbial community actively interacts with the barley grain and thus has great effects on the safety, technological, nutritional, and organoleptic properties of the final product. Depending on the nature and amount of microbes these consequences may be either deleterious or beneficial (Table 2).

1.3.1 Negative effects of microbes

Many microbial groups belonging to the indigenous barley community are destructive plant pathogens. Species of fusaria such as *F. graminearum* (teleomorph *Gibberella zeae*) are the most important plant pathogenic species worldwide, and cause *Fusarium* head blight (FHB) of wheat and barley and ear rot of maize (Steffenson 1998). *Fusarium*-damaged barley cannot be processed in the malting plant.

Table 2. Overview of reported negative and positive effects of microbes on the quality of barley and malt.

Negative effects	Positive effects
Plant diseases → yield reduction	Enhancement of grain germination (plant growth regulators such as gibberellin)
Inhibition of grain germination (water sensitivity, secondary dormancy)	Prevention of harmful microbes
Qualitative and quantitative changes in cereal components	Health-promoting compounds (vitamins, antioxidants etc.)
Metabolites causing process technical problems • organic acids causing variation in wort pH • slimes causing wort filtration problems • factors causing premature yeast flocculation (PYF) • gushing factors causing beer overfoaming	Production of hydrolytic enzymes contributing to malt modification • amylases • β-glucanases • proteases • xylanases
Toxic metabolites	
Allergens	

Most of the negative impacts of microbes have been related to rainy seasons during the harvest period leading to so-called weathered barleys, which are more seriously contaminated with bacteria and fungi (Bol & Huis in't Veld 1988, Flannigan 2003). The degree of weathering and the extent of invasion by microbes influence the seed vigour and the rapidity of grain germination (Etchevers *et al.* 1977). Occasionally, microbes are responsible for inhibited grain germination during malting. Van Campenhout (2000) reported that microbes in the grain tissues represent an inhibitory factor for barley respiration. It has been shown that especially aerobic microbes compete with barley for dissolved oxygen during the steeping phase, and uncontrolled multiplication of microbes may lead to poor germination (Briggs & McGuiness 1993, Doran & Briggs 1993, Kelly & Briggs 1992, 1993). Microbes are involved in phenomena such as water sensitivity of barley and post-harvest dormancy, which are detected as the inability of barley to germinate when placed under water (Doran & Briggs 1993, Kelly & Briggs 1992). Failure of grains to germinate in malting conditions is naturally a severe problem for a maltster.

The indigenous microbial community has been recognized as a significant factor causing variability in the malt batches (van Campenhout 2000). Microbial metabolism causes changes in cereal carbohydrates, lipids and proteins, and may therefore lead to quality failures. Uncontrolled degradation of barley components results in discoloration of grains and formation of off-odours and -colours (Flannigan 2003, Noots *et al.* 1999, Schildbach 1989).

Viable microbial cells originating from malt are destroyed at the latest by high temperatures during mashing and boiling in the breweries (O'Sullivan *et al.* 1999), but it is well known that microbial metabolites produced in the field or during malting may survive throughout the processing and have serious impacts later in the brewing process. Microbial communities have been shown to be responsible for the fluctuating organic acid levels of malt batches. The problem of variation in wort pH in different batches of malt, leading to inconsistent brewhouse performance, was identified partly as a microbiological problem by Stars *et al.* (1993) over a decade ago.

Some bacteria and also yeasts are known to produce extracellular polysaccharides (EPS) during the malting process, and these slimy compounds have been shown to cause problems during wort separation (Haikara & Home

1991, Kreisz *et al.* 2001). Mash filtration difficulties caused by split barley kernels were also identified as a microbiological problem (Haikara & Home 1991, Laitila *et al.* 1999). Frequent alternation between wet and dry conditions at a certain stage of barley ripening in the field occasionally leads to splitting of barley, in which the surface of kernel is broken and the barley endosperm is exposed to microbial attack. As little as 2–5% of split kernels in the malting barley batch may lead to severe wort separation problems in breweries. Moreover, dead malt-derived bacteria have been shown to cause visible haze in wort and in the final beer (Walker *et al.* 1997).

Brewers around the world have sometimes faced the problem of premature yeast flocculation (PYF) with some malt batches, i.e. the brewing yeast prematurely settles at the bottom of the fermentation tank leading to an incomplete fermentation and undesirable beer flavour (Blechova *et al.* 2005, van Nierop *et al*. 2006). Natural variation occurs between brewer's yeasts in sensitivity to PYF factors, some lager yeasts being more sensitive than others. The PYF phenomenon has been associated with fungal activity in barley. Breakdown of the husk arabinoxylans by fungal enzymes has resulted in the formation of factors inducing PYF (van Nierop *et al.* 2004). PYF factors can be produced in the field or generated during malting (Blechova *et al.* 2005, van Nierop *et al.* 2004). Blechova *et al.* (2005) reported that PYF tendency was also closely correlated with gushing tendency and was increased when barley was artificially inoculated with *F. graminearum* (teleomorph *Gibberella zeae*) and *F. culmorum*, whereas fungicide treatment of barley during the growth period reduced PYF tendency.

Contamination of the barley crop by fusaria or other filamentous fungi is of concern particularly in years when bad weather conditions favour the growth of gushing active and toxigenic species. Gushing is a term used to describe spontaneous overfoaming of packaged beer immediately on opening (Figure 7). Based on a recent German survey, over 50% of breweries have experienced gushing at least once (Niessen *et al.* 2007). The loss of image with the customer for a beer brand in cases of gushing may have significant economical impacts. Gushing is a very complex phenomenon, and it can at least partially be explained by the secretion of specific gushing factors by fungi which are present in malt or in other cereal-based raw materials applied in brewing (Amaha & Kitabatake 1981, Munar & Sebree 1997, Sarlin *et al.* 2005, Schwarz *et al.* 1996).

Recent studies have indicated that small, secreted fungal proteins called hydrophobins act as gushing factors (Haikara *et al.* 2000, Kleemola *et al.* 2001, Sarlin *et al*. 2005). They can be produced in the field or during malting. Hydrophobins are among the most important structural proteins found on the surfaces of fungal aerial structures such as hyphae, conidia and fruiting bodies (Kershaw & Talbot 1997). They play key roles in the development and in the interactions of fungi with the environment and other organisms such as plants. Hydrophobins react to interfaces between fungal cell walls and the air or between fungal cell walls and solid surfaces (Linder *et al.* 2005, Wessels 1996, 1997).

Figure 7 shows the two unwanted phenomena related to *Fusarium* growth during malting: beer gushing (7A) and mycotoxin production during malting (7B).

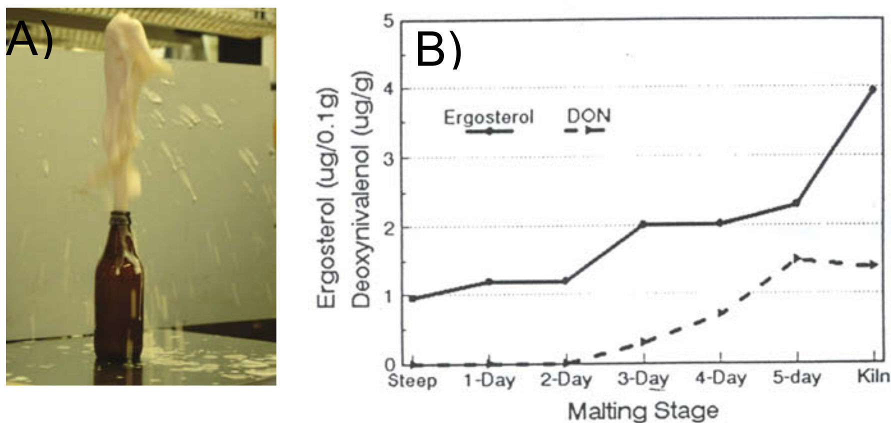

Figure 7. A) Beer gushing (reference Linder et al. 2005). B) Fungal biomass (ergosterol) growth and deoxynivalenol (DON) production during malting (reference Schwarz et al. 1995).

Many filamentous fungi are capable of producing toxic secondary metabolites, mycotoxins, in response to stressful conditions. The three main mycotoxigenic fungi associated with the cereal chain belong to the genera *Aspergillus*, *Penicillium*, and *Fusarium* (Sweeney & Dobson 1998). Mycotoxins are very stable compounds and can therefore survive throughout the processing and enter the final product (Schwarz *et al.* 1995, Scott 1996, Wolf-Hall & Schwarz 2002). Production of mycotoxins such as trichothecenes and zearalenone is probably

the most negative consequence associated with heavy contamination of barley and malt by *Fusarium* fungi (Haikara 1983, Munar & Sebree 1997, Schwarz *et al.* 1995, 1996, 2001). Production of *Fusarium* toxins during malting and their passage into beer have been demonstrated (Schwarz *et al.* 1995, Scott 1996). Water-soluble mycotoxins are largely removed during steeping of barley. However, due to mould growth during germination and the initial phase of kilning, additional toxins are sometimes produced during malting (Figure 7B). In addition, *Fusarium* toxins have been shown to disturb yeast metabolism during brewing (Boiera *et al.* 1999a, 1999b, 2000, Whitehead & Flannigan 1989). The degree of growth inhibition was dependent on the toxin concentration and the type of yeast strain and the length of fermentation (Boiera *et al.* 1999a, 1999b).

Microbes present in barley and malt or in grain dust, especially the spores of certain fungi, are also potent sources of allergens to the workers in malt houses and breweries. Diseases such as farmer's or maltworker's lung and brewer's asthma are results of allergic responses to high concentrations of inhaled spores (Flannigan 1986, Heaney *et al*. 1997, Rylander 1986).

1.3.2 Beneficial effects of microbes

Although microbes and their metabolites may have adverse impacts on malt properties and on subsequent brewing performance, the positive contribution of microbes on the malt characteristics is also significant.

Grain-associated microbes produce substances including hormones and enzymes which interact with the germinating barley during malting (Etchevers *et al*. 1977). Barley germination is metabolically regulated by a series of plant growth regulators. It is well known that many different microbes take part in the production of hormones which stimulate the grain germination. *Fusarium* fungi are known to produce gibberellins enhancing barley growth (Flannigan 2003, Haikara 1983, Prentice & Sloey 1960). Tuomi *et al*. (1995) reported that fungi and bacteria in the barley ecosystem contributed to the production of gibberellic acid, indole-3-acetic acid and abscisic acid (ABA).

Microbes in the malting ecosystem are also known to produce various types of antimicrobial factors in order to compete with other members of the diverse

microbial community (van Nierop *et al.* 2006). By microbiota management it is possible to enhance the growth of beneficial microbes which show antimicrobial potential (Haikara & Laitila 2001, Laitila *et al*. 2002, Lowe & Arendt 2004, Vaughan *et al.* 2001). Well-characterized barley and malt-derived bacteria and fungi with antimicrobial properties offer a potential alternative as natural, food-grade biocontrol agents. They can be applied as starter cultures in malting applications in which the use of chemical antimicrobials is considered undesirable (see Section 1.5.5).

Furthermore, microbes contribute to the nutritional value of malted cereals by removing antinutritive compounds and by enhancing the bioavailability of components such as minerals (Hammes *et al.* 2005). Several microbes such as yeasts have been shown to contribute to vitamin production in many cereal-based fermented products (Steinkraus 1998). So-called bioenrichment with natural microbes derived from cereal ecosystems has gained increasing interest in recent years. These characteristics are highly appreciated in the production of novel types of malt-based products with a healthy image.

More importantly, microbes in the malting ecosystem are producers of amylolytic, proteolytic and cell wall-degrading enzymes with positive effects on the malt characteristics (Bol & Huis in't Veld 1988, Hoy *et al*. 1981, Flannigan 1970, Flannigan & Dickie 1972, van Campenhout 2000, Yin *et al.* 1989). In some experiments the contribution of microbes to the barley β-glucanase pool has been estimated to be as high as 50–80% (Angelino & Bol 1990, van Waesberghe 1991). Furthermore, a substantial part of the malt xylanolytic activity originates from the indigenous microbial community. Van Campenhout (2000) reported that approximately 75% of malt xylanase activity was derived from microbes and only 25% from the grain. Barley- and microbe-derived hydrolytic enzymes play a key role in beer production by catalyzing the breakdown of biopolymers in malting and mashing.

1.4 Detection of microbes in the malting ecosystem

Early detection of changes in the microbial community is a significant component of quality control in the barley-malt-beer chain. For the maltster it is important to estimate and control microbial activities in order to obtain products

with predetermined malt specifications and suitable quality. However, the current microbial detection and identification approaches are too laborious and time-consuming to be used for routine process control. Furthermore, they often result in an incomplete picture of the true microbial diversity present. Therefore, there is a need for rapid and selective detection and quantification tools providing a reliable estimate of microbial dynamics in the malting process. Combination of different culture-dependent and -independent methods is often necessary in order to obtain a realistic view of the microbial ecology in a specific environment such as barley and the malting ecosystem. Table 3 compiles the benefits and limitations linked to current culture-dependent and -independent microbial community analyses.

1.4.1 Culture-dependent approach

After harvesting, the microbiological quality of malting barley is normally evaluated by visual and organoleptical inspection by the trader or maltster (Angelino & Bol 1990). Furthermore, the standard methods to assess microbial diversity are based on the enumeration and isolation of species growing on selective or non-selective growth media. Both direct and dilution plating are applied in barley and malting research (Flannigan 2003, Noots *et al.* 1999). Selected microbial isolates are then characterized and identified with phenotypic (physiological and biochemical) and genotypic (such as species-specific PCR, DNA fingerprinting, sequencing) approaches (Giraffa & Neviani 2001).

Currently, the standard methods for barley and malt analyses only include detection of fusaria, storage fungi and general field fungi (Abildgren *et al.* 1987, Gyllang *et al.* 1981, EBC Analytica Microbiologica 2001). The other microbial groups are not routinely monitored. Colony forming unit estimation is not reliable for filamentous fungi, since it tends to emphasise fungi which readily fragment or produce large numbers of spores. Therefore, filamentous fungi are generally determined by direct plating (Gyllang *et al.* 1981, Rabie *et al.* 1997, EBC Analytica Microbiologica 2001). The results are given as percentages of kernels contaminated with different mould genera, also known as the Mold Frequency Index (MFI) (Flannigan & Healy 1983). However, this approach only gives an estimation of the species present, not the degree of infection.

Table 3. Benefits and limitations of culture-dependent and -independent microbial community analyses.

Culture-dependent analysis		**Culture-independent analysis**	
Cultivation on selective and non-selective growth media **followed by** Phenotypic (physiological and biochemical) and genotypic (species-specific PCR, DNA-fingerprinting, sequencing) characterization and identification		Visual and organoleptic properties, microscopy, biomass, microbial metabolites (volatile compounds, toxins etc.), antibody techniques **or** Direct DNA/RNA approaches such as PCR (PCR-DGGE, RT-PCR, real-time PCR), hybridization (FISH), cloning/sequencing and transcriptional profiling	
Benefits	**Limitations**	**Benefits**	**Limitations**
+ microbes available for further application	- time-consuming and laborious	+ detection of unculturable microbes	- microbial isolates not available
+ isolate represents a certain species	- many microbes unculturable	+ genetic diversity in real environment	- requires genetic information (sequence data)
+ indicates viability		+ specific groups within complex ecosystems	- most techniques also detect dead cells
		+ *in situ* metabolic activity	

FISH; fluorescence in situ hybridization, DGGE; denaturing gradient gel electrophoresis, RT-PCR; reverse-transcriptase-polymerase chain reaction

The great advantages of the culture-dependent approach are that individual microbial isolates can be identified, and these are then available for further characterization and exploitation. The major disadvantage is that only few microbes in nature can be isolated in pure cultures (Amann *et al*. 1995). This is mainly due to the current lack of knowledge of the growth conditions under which certain microbial populations live in their natural habitat. Therefore, only certain microbial groups can be assessed by a culture-dependent approach. In addition, fast-growing organisms can overgrow the slower species in the plate assays, thus hindering their detection.

1.4.2 Culture-independent approach

New powerful analytical tools enable us to investigate complex microbial ecosystems in their natural environment without the need to isolate and culture individual components (Giraffa & Neviani 2001). Generally these are nucleic acid-based methods, although direct microscopy and analyses of microbial metabolites such as mycotoxins can also be included in this category. Furthermore, immunochemical procedures have been established for the detection of field and storage fungi such as fusaria in barley and malt samples (Vaag 1991). Direct DNA/RNA extraction approaches from environmental samples, coupled with polymerase chain reaction (PCR) amplification and community profiling techniques have become widely applied in studying microbial ecology in complex environments (Ercolini 2004, Muyzer & Smalla 1998). PCR-based methods are more rapid and convenient than the traditional culture-based methods. Furthermore, they also allow the detection of non-culturable species. PCR-primers can be targeted to specific microbial groups, and therefore it is possible to monitor the presence, succession and persistence of certain microbial populations within a complex ecosystem. Recently, diagnostic and quantitative PCR assays have been developed to detect and quantify individual pathogenic fungi within polymicrobial infections, and to detect trichothecene-producing fusaria in barley and malt (Bluhm *et al.* 2004, Nicholson *et al.* 2003, Sarlin *et al*. 2006).

At present, denaturing gradient gel electrophoresis (DGGE) is perhaps the most commonly used culture-independent fingerprinting technique for studying the response of microbial community dynamics. In DGGE, PCR-amplified DNA products with the same length but different sequence can be separated on a gel, resulting in unique fingerprints of environmental DNA samples (Muyzer & Smalla 1998). Universal PCR-DGGE targeting to ribosomal genes of bacteria and fungi detects the predominant species of a community without discriminating living from dead cells or cells in a non-culturable state. The main populations, which constitute 90–99% of the total community, are displayed in the profiles. This technique has also demonstrated its potential in food-related ecosystems (Ercolini 2004, Giraffa & Neviani 2001) and has been applied in beverage fields such as whisky production (van Beek & Priest 2002, 2003) and wine fermentations (Lopez *et al.* 2003). Advantages and disadvantages of PCR-DGGE were reviewed by Ercolini (2004) and Muyzer (1999).

1.5 Management of microbes in the malting ecosystem

Production of high quality malt ingredients and beverages relies on good malting and brewing practices in the entire barley-malt-beer chain.

1.5.1 HACCP and hygiene in malt production

Malting is classified as a food process, and therefore industrial protocols legislated for food business operators are applied in malting houses. The HACCP (Hazard Analysis and Critical Control Points) concept is an effective system for controlling both food safety and quality and it has also been implemented in the malting and brewing industry (Rush 2006, Davies 2006). HACCP involves identifying all points in the manufacturing process where biological, chemical and physical hazards could occur and then controlling and monitoring those risks. It also covers the cereal co-products such as malt sprouts and spent grains of the malting and brewing process used as animal feed.

Every process step in malting can be a source of additional microbes, and therefore good malting plant hygiene is essential. Although malting is not an aseptic process, hygiene standards are set up in the malting plants as well as around the surrounding environment and included in the HACCP standards (Davies 2006). Empty silos are cleaned to remove the grain residues and occasionally fumigated in order to eliminate the contaminants. Sanitation of empty malting vessels and air-conditioning systems is carried out in order to avoid harmful process contaminants. Preventive measures are vitally important in maintaining the quality of malting barley and in assuring safety throughout the malting and brewing process.

1.5.2 Importance of barley and malt storage

Control of grain safety in silos and in transport is crucial with respect to malt quality. Water is the most important single factor limiting microbial growth. Immediate drying of the barley crop after harvest below a_w 0.7 efficiently restricts the growth of most fungi (Flannigan 2003). During storage the barley moisture content is in equilibrium with the moisture content of the air (Kunze

1999). Therefore, grains may be further dried or they can absorb water from the surrounding air during storage. The storage life of stored grains is increased by cooling. Barley and malt should always be stored in a dry and cool environment to avoid the potential risks associated with fungal growth and possible mycotoxin accumulation.

1.5.3 Quality of the incoming barley

Grain deterioration due to plant pathogenic fungi often leads to poor kernel filling. Barley lots with poor quality can easily be noticed on the basis of decrease of grain weight relative to the control (Chelkowski 1991). The method of weighing 1000 kernels is routinely applied in quality control and heavily infected barley lots are discarded before entering the malting process. Furthermore, grading of barley prior to processing is an important step to remove cereal matter that is not suitable for malting and to reduce the microbial load. Significant amounts of contaminated kernels can be rejected by barley grading in which the barley is sorted into fractions of even kernel size in order to obtain homogenous malt (Kunze 1999). It has been shown that high amounts of mycotoxins are often observed in small kernels. Therefore, by rejection of the smallest sized kernels (< 2.5 mm), a significantly reduced level of *Fusarium*-contaminated grains and mycotoxins in barley can be obtained (Perkowski 1998).

1.5.4 Chemical and physical means

Several chemical microbicidic agents are effective in eliminating bacteria and fungi. Fungicides are occasionally applied in the field in order to protect the barley against plant pathogens. However, they may not be applied after the harvest. Recently, considerable research effort has been directed towards the development of novel compounds for plant disease control in order to minimize the use of chemicals and to reduce the resistance risk of field fungi (Gullino *et al.* 2000). It should be noted that application of fungicides during the growth period of barley may also have an impact on the microbial communities during malting and on the subsequent malt quality. Havlova *et al.* (2006) reported that application of some fungicides during barley cultivation increased the gushing

potential of the final malt. In addition, suppression of field fungi with fungicides increased pentosan and β-glucan levels in malts, with negative impacts on wort and beer properties. As a consequence, research has been directed towards developing more natural means for balancing the microbial communities in barley and during malting.

The treatments carried out during malting should not significantly influence the seed vigour. Various common practices are routinely applied to reduce adverse effects of microbes during malting, especially during the steeping phase, such as changing the steeping water in order to remove microbes and leached nutrients, balancing the temperature or modifying aeration (Briggs & McGuinness 1993). Furthermore, steep water must be warm enough to allow rapid water uptake and germination of the grains, but cool enough to avoid extensive microbial growth. Therefore, steeping is normally carried out at 10–20 °C (Pyler & Thomas 2000). It is also important to provide sufficient aeration and to pulse the circulation throughout the immersion period in order to keep the grains moving and to avoid anaerobic, hot pockets in the grain bed which would lead to increased microbial activity and poor grain germination (Davies 2006).

Doran and Briggs (1993) reported that by acidifying the grain in the first steep the adverse effects of aerobic microbial growth could be greatly reduced. Papadopoulou *et al.* (2000) suggested that fungal proliferation could be restricted by adding hop beta-acids into the malting process. Moreover, they demonstrated that the growth of fungi was inhibited by washing barley first with sodium hypochlorite (alkaline wash) followed by an acid wash with hypochloric acid. However, the feasibility and safety of acid treatments in large scale remains to be confirmed. Disinfectants have occasionally been added to steeping water in order to intensify the washing effect during steeping. Although different additives may effectively improve processing, their use in industrial processes is often limited by the legislation. Furthermore, the industry has a strong emphasis towards natural processing without chemicals (Olkku *et al.* 1992)

It is a well-known fact that several filamentous fungi, especially field fungi such as fusaria, are sensitive to heat. High-temperature treatments have been shown to effectively reduce the viable fungi on cereal grains, although it does not eliminate the preformed mycotoxins (Kristensen *et al.* 2005). Olkku *et al.* (2000) reported an invention in which the mould contamination of barley was

effectively reduced by exposing grains to heat (60–100 °C) for 0.5–3 s prior to the malting process. Heat treatment of barley notably decreased the *Fusarium* contamination without influencing grain germination. Moreover, it significantly reduced mycotoxin production during the malting process and alleviated the gushing tendency (Olkku *et al.* 2000).

Kottapalli *et al.* (2003) studied hot-water treatments for reducing fusaria in malting barley. They showed that soaking barley with water at 45 °C for 15 min resulted in a significant reduction of *Fusarium* contamination without influencing grain germination. The same effect was obtained by soaking at 50 °C for 1 min. A great advantage of hot water treatments was that some water-soluble mycotoxins could be washed out of the grain. Briggs (2004) reported that short exposures to hot water, even at 100 °C for 5 s were advantageous with respect to microbiological safety and grain germination. Thus grains would be washed and surface-sterilized prior to steeping.

In addition to heat treatments, electron-beam irradiation of barley has been reported to be an effective, non-chemical means for reducing fusaria. For dry *Fusarium*-infected barley, an irradiation dose of > 4 kGy was required to obtain *Fusarium* reduction (Kottapalli *et al*. 2003, 2006). Although irradiation had no effect on the preformed mycotoxins, it greatly reduced the mycotoxin production during malting (Kottapalli *et al.* 2006). The advantage of irradiation was that the grain treatment could be carried out either prior to or after the storage. It could also help in the maintenance of quality by eliminating insect infestation. Insects such as beetles are the principal vectors of microbes in plant ecosystems (Suh & Blackwell 2004). Grain germination was not significantly influenced up to a dosage of 8 kGy (Kottapalli *et al.* 2003).

Residues and undesirable reaction products in germinating barley and in the subsequent malt are of concern especially with chemical treatments, since they may have a negative impact on malt properties and yeast fermentation performance. Furthermore, precautions must be taken as some of the antimicrobial treatments in sublethal doses may stimulate the production of harmful metabolites such as gushing factors and mycotoxins. Malt-derived microbes, especially lactic acid bacteria and certain fungi, offer a potential alternative as natural, food-grade biocontrol agents. Natural biocontrol agents are attractive as they have a better public image and they could potentially be

used as starter cultures in bioprocesses in which the use of chemicals is considered undesirable. Some of the potential LAB and fungal strains studied in malting applications are listed in Table 4.

1.5.5 Lactic acid starter cultures in malting

LAB are widely applied in the food and feed industry. The success of LAB is due to their ability to improve safety, flavour, nutritional value and structure of the products (Salminen & von Wright 2004). Several investigations have also been conducted to examine the antimicrobial properties of LAB isolates from barley and malt and their potential against microbial contaminants in malting and brewing (Hartnett *et al*. 2002, Laitila *et al.* 2002, O'Mahony *et al.* 2000, Niku-Paavola *et al.* 1999, Vaughan *et al.* 2001, 2003, 2004). The microbistatic and/or microbicidic action of LAB is based on both the competition for nutrients and production of various antimicrobial compounds such as organic acids, hydrogen peroxide, bacteriocins and low-molecular weight antimicrobials (Ouwehand & Vesterlund 2004). Recently, Lowe and Arendt (2004) reviewed the potential of LAB in malting and brewing applications.

In addition to their antimicrobial potential, the use of LAB in malting has led to improvements in malt properties. Malt-derived thermophilic LAB such as *Lactobacillus delbrueckii* or *L. amylovorus* strains have traditionally been used in the production of biologically acidified malt, mash or wort (Back 1988, Englmann & Reichert 1991, Lewis 1998, Narziss & Heiden 1971). Biological acidification has been practised for centuries in brewing applications in which Reinheitsgebot i.e. German Purity Law is strictly enforced. The ultimate goal is to establish a defined pH level in the mash or wort without using additional acids for pH adjustment. In addition to improved microbiological stability, biological acidification has contributed to the technological and organoleptic properties of malt, wort and beer (Lewis 1998, Pittner & Back 1995, Lowe *et al.* 2004, 2005b).

Table 4. Microbial cultures utilized in malting applications.

Microbe	Strain	Main Purpose	References
LAB			
Lactobacillus amylolyticus	TMW1.268	Biological acidification (BA)	Lowe *et al*. 2005b
L. amylovorus	FST 1.1	BA	Back 1988, Lowe *et al.* 2005a
L. delbrueckii		BA	Narziss & Heiden 1971, van Waesberghe 1991
L. plantarum	TMW 1.460	BA	Lowe *et al*. 2005a
	VTT E-78076	Restriction of fusaria and Gram-negative bacteria	Haikara *et al.* 1993, 1994, 1995, 2001
		Enhancement of malt processability	Laitila *et al.* 1997, 1999, 2002
Pediococcus pentosaceus	VTT E-90390	Restriction of fusaria and Gram-negative bacteria	Haikara *et al.* 1993, 1994, 1995, 2001
		Enhancement of malt processability	van Campenhout 2000
	L7230	Bacteriocin production	van Campenhout 2000
Filamentous fungi			
Rhizopus oligosporus		Enhancement of malt modification	Coppens *et al.* 1996, Noots *et al.* 2003, Dufait & Coppens 2002
Yeast-like fungi			
Geotrichum candidum	IFBM	Inhibition of toxigenic fungi	Boivin & Malanda 1996, 1997, Boivin 2002
	S1	Extract yield and improvement of wort filtration	Dziuba & Fosczynska 2001

Earlier studies at VTT revealed two potential LAB strains: *Lactobacillus plantarum* VTT E-78076 (isolated from beer) and *Pediococcus pentosaceus* VTT E-90390 (isolated from barley) (Haikara & Mattila-Sandholm 1994, Haikara *et al.* 1993). These two strains added into the steeping waters of barley restricted the growth of harmful bacteria causing wort filtration problems and of *Fusarium* fungi during the malting process (Haikara & Laitila 1995, Laitila *et al*. 1997, 1999, 2002). Previous studies also showed that LAB starter cultures added into the steeping contributed to the enzyme potential of malt and to wort separation performance (Haikara *et al*. 1993, Haikara & Laitila 1995, 2001).

1.5.6 Fungal starter cultures in malting

Barley-associated filamentous fungi are known to produce various types of hydrolytic enzymes. Recently, a starter technology based on the use *Rhizopus oligosporus* was developed for malting applications with a particular aim to compensate for deficiencies in malt cell wall modifying enzymes (Coppens *et al.* 1996, Dufait & Coppens 2002, Noots *et al.* 2001, 2003). *R. oligosporus* S46 produced β-glucanase, xylanase and proteases, whereas no increase in starch degrading power was detected. Dormant spores needed 4–5 h activation prior to addition to the first steeping water (Dufait & Coppens 2002). Invasion of the starter culture through the outer layers of the kernel was the rate-limiting step for degradation of the cell walls of the starchy endosperm (Noots *et al.* 2003). However, fungal enzymes produced during malting were also active during the mashing stage and notably improved lautering performance was observed after *Rhizopus* addition to the malting process.

It has been reported that several ascomycetous and basidiomycetous yeasts derived from plant ecosystems have strong antagonistic activity against various fungal pathogens (Blakeman & Fokkema 1982). Several yeast strains have successfully been applied to prevent pre-and post-harvest fungal diseases of fruit and vegetables (Boekhout & Robert 2003) and to control spoilage moulds during storage of high moisture feed grains (Druvefors *et al.* 2002, Petersson & Schnürer 1998). However, rather little is known about the antifungal potential of the yeasts derived from the malting ecosystem. Boivin and Malanda (1996, 1997) showed that a *Geotrichum candidum* (teleomorph *Galactomyces geotrichum*) isolate derived from a malting process restricted fungal growth and

prevented mycotoxin formation during malting. Starter suspension was either sprayed over the barley before steeping or mixed with the water at the first steep (Boivin 2002). Furthermore, the treatment with *G. candidum* improved malt modification.

1.5.7 Combination of various treatments

Controlling the malting ecosystem is extremely challenging, because the complex microbial communities are associated with an active grain matrix and the procedures carried out during malting should not have negative consequences on the barley metabolism. The combination of several mild treatments could result in a successful strategy for management of microbes in complex ecosystems such as malting. The concept of hurdle technology is widely applied in food preservation and it aims at improving the total quality of foods by applying gentle, multitarget preservative factors (hurdles) with synergistic effect (Leistner 2000). The hurdles are intentionally combined to improve the microbiological stability, sensory and nutritional properties of the products. The most important hurdles used in food preservation systems are temperature (high and low), low water activity (a_w), acidity (pH), low redox potential (Eh), preservatives (nitrite, sorbate, sulfite) and competitive microbes (such as LAB) (Leistner 2000).

The hurdle concept that exploits synergistic interactions between various treatments is common in malting and brewing practices (Vaughan *et al.* 2006). Briggs (2002) suggested that the first steeping, when the grains require less oxygen, could be almost anaerobic. Minimal aeration could be used to loosen the grain bed. In addition, the steep water could be made slightly acidic. Anaerobiosis and acidity limit microbial multiplication. Furthermore, he suggested that extra rinsing prior to germination would be beneficial in removing microbes as well as solids. The first steeping water of 21.5 tonnes of barley contained about 90.4 kg of solids and with further rinsing 14.1 kg more solids were removed (Briggs 2002).

Well-characterized microbial mixtures consisting of barley and malt-derived bacteria and fungi offer an additional measure to guarantee microbial safety and to tailor the malt properties. Biocontrol agents are often introduced to various

applications as single cultures. Recently, research has also been directed to combining several biocontrol agents or linking microbial cultures with other preservation methods. Yeast and LAB often occur together in plant-based bioprocesses, and synergistic interactions between these two groups are utilized in many cereal fermentations (Boekhout & Robert 2003). To our knowledge LAB and yeasts or filamentous fungi have not yet been combined in malting applications. Microbial cultures could also be connected with other physical and chemical treatments. Olkku *et al.* (2000) reported that the heat treatment of barley prior to malting followed by *L. plantarum* E76 addition into the steeping waters was found to be an effective combination in controlling *Fusarium* growth in malting.

Selective control of microbial populations in various steps could be a successful strategy to suppress the harmful organisms such as gushing-active and toxigenic fungi and to simultaneously enhance the beneficial organisms such as microbes contributing to malt modification and malt brewhouse performance. However, in order to exploit novel technologies in malting, more knowledge is needed on the complex interactions between the abundant microbes of barley and grain metabolism.

Van Waesberghe (1991) nicely condensed the idea of microbiota management in malting with microbial cultures:

"You cannot stop the microbes growing. So if you don't beat them, join them."

The ultimate goal is to assure and improve the safety, functionality and economy of the malting process.

2. Aims of the study

Grain-microbial interactions are an integral part of the malting process. Management of microbes in malting is a complex interactive process, and both microbial and barley activities must be considered simultaneously. More knowledge is needed on the impacts of specific microbial populations within the malting ecosystem. The hypothesis of this dissertation was that the diversity and the role of microbes in the malting ecosystem is more substantial than previously anticipated and needs further characterization. The ultimate goal was to broaden understanding of the relationships between microbial communities and the germinating barley during malting. Furthermore, the study aimed to create possibilities for tailoring of malt properties with well-characterized microbial cultures. A profound understanding of the microbial communities is essential in order to establish the origin and the impacts of beneficial and detrimental microbes and their management.

The specific aims of this study were

- to introduce new approaches to assess and modify population dynamics in the malting ecosystem (Papers I–IV)
- to investigate the impacts of indigenous bacterial and fungal communities present in good-quality barley on grain germination and malt properties (Papers I, II)
- to evaluate the potential of lactic acid bacteria in the management of microbial communities and in the enhancement of malt processability (Papers II, IV)
- to investigate the diversity and the impacts of yeasts and yeast-like fungi in the malting ecosystem (Papers III, IV)
- to combine lactic acid bacteria with antagonistic yeasts in order to prevent the growth of gushing-active fungi in malting and to enhance the usefulness of starter technology in the tailoring of malt properties (Paper IV).

3. Materials and methods

The samples and experimental protocols are described only briefly in this section. For more detailed information see the original publications (Papers I–IV).

3.1 Microbial cultures

Bacteria, yeasts and filamentous fungi used in this study originated from the VTT Culture Collection (Papers II, IV). All strains had been isolated from malting or brewing processes. Microbes were cultivated according to standard laboratory practices on media recommended by Suihko (1999). Yeasts and yeast-like fungi isolated and identified from the industrial maltings (Paper III) were deposited in the VTT Culture Collection. The strain list with code numbers is presented in Paper III.

3.2 Malting trials

All the malting experiments were carried out with two-row barley varieties (*Hordeum vulgare* L, *Poaceae*) (Papers I–IV). Before malting, barley samples were sieved to remove grains smaller than 2.5 mm. Details of the malting programs are presented in Papers I–IV.

3.2.1 Laboratory scale malting trials (Papers I and IV)

Barley samples (300–1000 g) were malted in a specially designed computer controlled micromalting equipment with a separate drum for each sample (Hulo Engineering, Helsinki, Finland). The concentration of volatile compounds in the head space of each malting drum was analyzed continuously using a Fourier Transform Infrared Spectroscopy (FTIR) multicomponent gas analyzer Gasmet® (Temet Instruments ltd, Helsinki, Finland) with a heated, flow-through, 5 m path length sample cell.

3.2.2 Pilot scale malting trials (Paper II)

Batches of 25 kg barley were steeped in an automated conical steeping vessel (Inssitiimi, Turku, Finland). After the steeping, samples were transferred to an automated pilot germination/kilning unit (designed at VTT). Rootlets were removed in an ejector-feeded grain pre-cleaner (Kongskilde pre-cleaner KF-12/FRL10, Kongskilde, Denmark).

3.2.3 Industrial malting trials (Paper III)

Four industrial lager malt production runs were monitored during the year 2002.

3.2.4 Addition of antimicrobials or microbial cultures into steeping

Table 5 shows the antimicrobial treatments carried out in laboratory or pilot scale malting experiments. In order to study the impact of microbial communities on grain germination and malt properties, the growth of bacteria and/or fungi was suppressed at the beginning of steeping by adding various antimicrobial mixtures (Paper I). Antimicrobial treatments were selected on the basis of their direct action on microbial metabolism without disturbing grain activity (Doran & Briggs 1993, Gaber & Roberts 1969, Kelly & Briggs 1992, van Campenhout *et al.* 1998, 1999). Antimicrobial treatments were compared to treatment with 0.05% hydrogen peroxide (H_2O_2) in the first steep. H_2O_2 is an active oxygen source that can readily diffuse into plant tissue and is generally known to enhance the germination of dormant barley.

Lactobacillus plantarum VTT E-78076 (E76) and *Pediococcus pentosaceus* VTT E-90390 (E390) were added into the steeping water of normal malting barley in order to balance the microbial community and enhance malt processability (Paper II). Furthermore, we investigated the effects of chemically acidified, unfermented MRS culture broth on the microbial community and malt quality (Paper II). The antifungal potential of *Pichia anomala* VTT C-04565 (C565) yeast strain was studied with naturally infested barley showing gushing activity. *P. anomala* C565 was also combined with *L. plantarum* E76 (Paper IV).

Table 5. Antimicrobial treatments performed in the malting experiments.

Acronym of treatment	Addition to steeping water	Primary goal	Paper
Antibac	I steep: 100 ppm chloramphenicol, 100 ppm chlortetracycline	Suppression of bacteria	I
Antimix	I steep: 100 ppm amphotericin B, 400 ppm nystatin, 800 ppm penicillin G, 400 ppm polymyxin B, 800 ppm streptomycin sulphate	Suppression of both bacteria and fungi	I
Bacmix	I steep: 800 ppm penicillin G, 400 ppm polymyxin B, 800 ppm streptomycin sulphate	Suppression of bacteria	I
Antifung	I steep: 100 ppm amphotericin B, 400 ppm nystatin	Suppression of fungi	I
L. plantarum E76	I and II steep: LAB culture, including cells and spent medium, 120 ml/kg barley	Balancing of the microbial community, enhancement of malt processability	II
P. pentosaceus E390	- “ -	- “ -	II
MRS-LA	120 ml/kg barley unfermented MRS broth without glucose and supplemented with 2.5% lactic acid	Acidification of steeping water	II
P. anomala C565	yeast cells 10^6 cfu/ml steeping water	Suppression of fusaria	IV
L. plantarum **E76 +** ***P. anomala*** **C565**	I steep: 120 ml/kg E76 culture and II steep: yeast cells 10^6 cfu/ml	Suppression of fusaria and enhancement of malt processability	IV

3.3 Detection, identification and characterization of the microbial communities

The methods applied to study the microbial communities in the malting ecosystem are summarized in Table 6.

Table 6. Experimental procedures used for characterization of the microbial communities.

Analysis	Paper	Ref.
Enumeration of microbes		
Total aerobic heterotrophic bacteria	I, II, IV	
Psedomonas spp.	I, II, IV	
Lactic acid bacteria	I, II, IV	
Yeasts	I–IV	
Fusarium fungi	I, II, IV	1, 2
Field fungi	I, II, IV	1
Molecular typing and identification		
Total DNA extraction from grains	I	3
DNA extraction from yeast pure cultures	III	3
PCR-DGGE targeting to the bacterial 16S rRNA gene	I	4
PCR fingerprinting of yeasts with M13 primer	III	5
Partial 16S rRNA gene sequencing (bacteria)	I	
Partial 26S rRNA gene sequencing (yeasts)	III	6
Production of extracellular hydrolytic enzymes		7
Amylase, plate-screening assay	III	
β-Glucanase, plate-screening assay	III	
Cellulase, plate-screening assay	III	
Xylanase, plate-screening assay	III	
Antifungal potential of yeasts		
Dual-culture overlay assay	IV	
Fungal hydrophobins	IV	8
FESEM microscopy		9

References: 1) EBC Analytica Microbiologica 2001, 2) Abildgren *et al.* 1987, 3) see Section 3.3.1, 4) Mättö *et al.* 2005, 5) Andrighetto *et al.* 2000, 6) Kurtzman & Robnett 1998, 7) see Section 3.3.2, 8) Sarlin *et al.* 2005, 9) see Section 3.3.3.

3.3.1 DNA extraction protocols for barley and malt

Total genomic DNA (including microbial and plant DNA) from duplicate ground samples (0.1 g) was extracted with FastDNA®Spin Kit for Soil (Q-Biogene, Carslbad, CA, USA) according to the manufacturer's instructions with the modification that the samples were homogenized with a FastPrep cell disrupter (FP120, QBiogene, Carslbad, CA, USA) at 6.0 m/s for 30 s four times (Paper I). In addition, DNA from ground barley and malt samples was extracted with NucleoSpin®Plant-kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instruction. We used three different extraction protocols: the genomic DNA elution protocol for plants without (2) and with RNase treatment (2+RNase) and with the CTAB procedure including mechanical disruption of the samples for 2 min at 5.5 m/s (2+mechanical treatment). In addition, we tested the rapid DNA preparation protocol as described by Kulik *et al*. (2004), in which the DNA was extracted with alkaline-detergent buffer from wheat samples for the diagnosis of fusaria.

In addition to grain samples, DNA was extracted from the malt-derived indicator microbes, which are known to be difficult to lyse due to their complex cell walls and capsules. *Leuconostoc citreum* VTT E-91451 (E451) was grown in MRS broth (Oxoid Ltd., Basingstoke, Hampshire, UK) in anaerobic conditions at 30 °C for 2 days. *Rhodotorula glutinis* VTT C-92011 (C11) was cultivated in YM-broth (Difco Laboratories, Detroit, USA) at 25 °C for 2 days. The cells were collected by centrifugation at 13 000 rpm for 3 min. *Fusarium graminearum* (teleomorph *Gibberella zeae*) VTT D-95470 was cultivated on potato dextrose agar plates at 25 °C for 7 days (Difco Laboratories). Fungal biomass (including mycelium and spores) was harvested from the plates with a bacteriological spreader. 0.1 g of the microbial cell mass was used for DNA extraction.

DNA-samples were analyzed by electrophoresis in 1% agarose gel (Cambrex Bio Science Rockland Inc., Rockland, USA) in 0.5 x TBE buffer at 120 V for 30 min. In addition, genomic DNA samples were determined with an Eppendorf photometer.

3.3.2 Production of hydrolytic enzymes by filamentous fungi

In addition to yeasts, the production of amylase, β-glucanase, cellulase and xylanase by filamentous fungi was studied with a plate-screening assay as described in Paper III. Both field and storage fungi (19 different strains) were included in the test panel. Fungal strains were cultivated on duplicate enzyme assay plates at 25 °C for 5–7 days. The strains are presented in Table 9.

3.3.3 FESEM microscopy of the grain samples

Grain samples for Field Emission Scanning Electron Microscopy (FESEM) were derived from the native barley and from the laboratory scale malting experiments after two days of steeping. Control samples were steeped in water and starter samples were derived from the malting experiments in which *L. plantarum* E76 was added into the first steeping water and *P. anomala* C565 to the second steeping water (Paper IV). Grains were stored at −20 °C prior to analyses. For microscopic analyses, 10 healthy looking kernels were selected randomly. FESEM samples were analyzed at the University of Helsinki, Department of Applied Chemistry and Microbiology. Grain samples were cut into half along the ventral furrow. Samples were fixed and analyzed as described by Raulio *et al.* (2006), except that the samples were coated with platinum-palladium in a vacuum coater. Several areas between the testa and the outer epidermis were examined.

3.4 Barley, malt and wort analyses

The methods applied for barley, malt and wort analyses are presented in Table 7.

Table 7. Experimental procedures used for barley, malt and wort analyses.

Analysis	Method	Paper	Ref.
Barley			
Moisture content	EBC 3.2	I–IV	1
Protein content	EBC 3.3.1	I–IV	1
Germination capacity	EBC 3.5.1	I–IV	1
Germination energy	EBC 3.6.2	I–IV	1
Grain germination		I, II, IV	
Germinated grains counted daily		I, II, IV	
CO_2 and ethanol from headspace		I, IV	2
Grain alcohol dehydrogenase (adh)		I, IV	2, 3
α-Amylase	Megazyme CER	I	2
Enzyme activities in malts			
α-Amylase	Megazyme CER	I	
Endogenous β-glucanase	Megazyme MBG	I, II, IV	
Microbial β-glucanase	Megazyme MGB	I, II, IV	
Xylanase	Megazyme XYL	I, II, IV	
Malt and wort analyses			
Malt friability	EBC 4.15	I, IV	1
Malt modification, Calcofluor	EBC 4.14	I, II, IV	1
Malt gushing potential		IV	4, 5
Congress mashing		I,II	1
Extract content	EBC 4.5.1	I, II	1
Wort colour	EBC 4.7.2	I, II	1
Free amino nitrogen (FAN)	EBC 4.10	I, II	1
Soluble nitrogen, Kjeldahl method	EBC 4.9.1	I, II	1
Wort β-glucan content, fluorimetric	EBC 4.16.2	I, II	1
Wort viscosity	EBC 4.8	I, II	1
Filtration rate, time needed to collect 300 ml of filtrate		I, II	1
High gravity mashing		II, IV	6
Büchner filtration		II, IV	6
Wort analyses as above	EBC	II, IV	1

References: 1) Analytica EBC 1998, 2) Wilhelmson *et al.* 2006, 3) Crawford 1967, 4) Vaag *et al.* 1993, 5) Haikara 1980, 6) Sjöholm *et al.* 1994.

4. Results and discussion

Microbes have a decisive role in the barley-to-beer chain. It is nowadays accepted that microbes are required in the production of high quality malt. Due to several unwanted properties, such as production of mycotoxins, filamentous fungi have attracted more attention during recent years, whereas less attention has been paid to the role of bacteria and yeasts in grain germination and malt quality. In this study the impacts of both bacterial and fungal communities on barley grain germination and on malting properties were investigated. Furthermore, the yeast community in the industrial malting ecosystem was thoroughly studied. In addition, this study was carried out to evaluate the potential of malt-derived microbes as natural, food-grade biocontrol agents. The ultimate aim was to provide useful information on microbial ecology in the malting ecosystem, which could be utilized when adapting new strategies for microbiota management or when designing new malt products.

4.1 Complex associations of microbes in the malting ecosystem (Papers I–IV)

This study showed that the microbial communities within the grain matrix were well protected. Although powerful antimicrobial agents were applied during malting they had surprisingly little effect on the viable microbial counts (Paper I, Figure 1). Some species were located deeper in the husk layers and were not necessarily influenced by the heat during kilning or by the external addition of antimicrobial agent or biocontrol agents (Papers I–IV). FESEM microscopy of the steeped barley samples revealed complex microbial biofilms in the seed-coat tissues (Figure 8A). Microbial cells and fungal hyphae were distributed throughout the layers between the husk and outer epidermis. Microbial cells were connected to the grain surfaces and to each other with specific adhesion threads forming tight networks inside the grain layers (Figure 8B).

Steeping of barley promoted microbial growth and also production of exopolysaccharides (EPS) and other exopolymeric substances. As seen from Figure 8A, the microbial cells in steeped barley samples were largely embedded in a thick slime matrix. Exopolymeric material protected the cells and aided the

microbial colonization to barley tissues. Furthermore, EPS could be used as a substrate for microbial growth. Biofilm-grown cells have shown increased resistance to antimicrobials (Costerton *et al.* 1987, Morris & Monier 2003). Complex biofilms could also explain the weak effects of the antimicrobial treatments applied in this study.

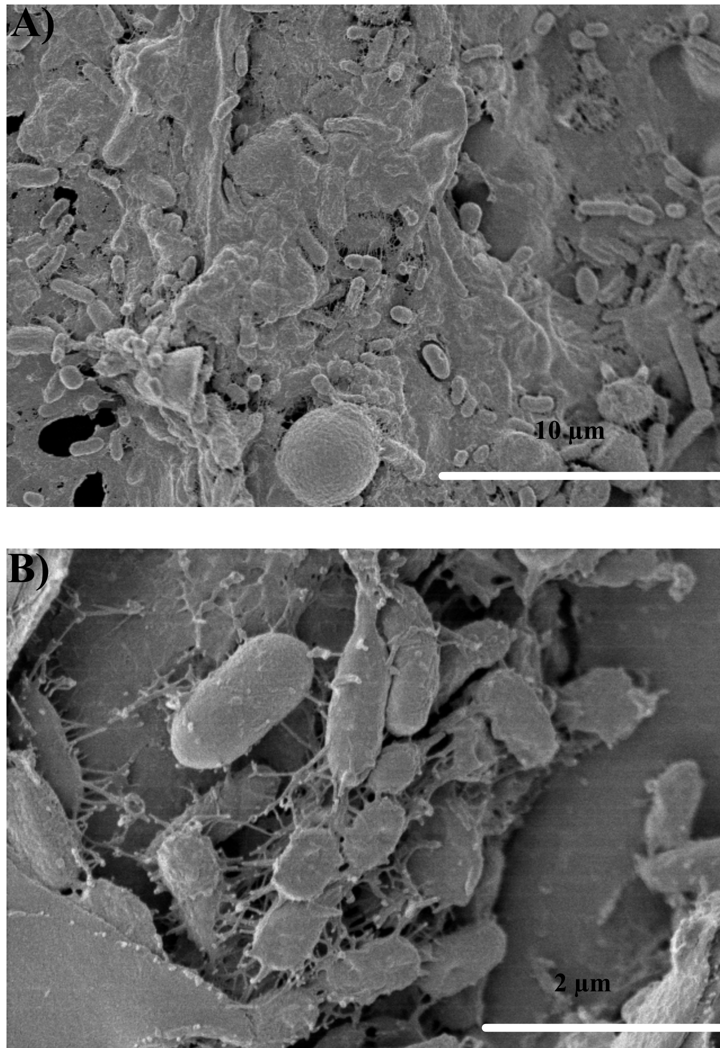

Figure 8. A) FESEM micrographs of the steeped grains show 2 day old biofilms in the outer layers of barley. Microbial cells embedded in the slime matrix. B) Bacterial cells connected to each other and to barley surfaces with adhesion threads. Pictures Mari Raulio, University of Helsinki & Arja Laitila, VTT.

Furthermore, this study revealed that reduction of one microbial population within the complex ecosystem led to the increase of non-suppressed populations (Papers I, II). We showed that suppression of Gram-negative bacteria with antibacterial antibiotics (Paper I) or with lactic acid starter cultures (Paper II) led to increased yeast growth. A decline in competitive microbes created more opportunities for other populations. Competitive interaction was also observed between *Fusarium* and *Alternaria* fungi (Paper II). A statistically significant increase of *Alternaria* fungi was recorded when the fusaria were suppressed with LAB cultures. Similar results of antagonistic interaction between *Alternaria* and *Fusarium* fungi in the barley grains have been reported by Haikara *et al.* (1977), Andersen *et al.* (1996) and Lacey (1989). Our results are also supported by the findings of Kottapalli *et al.* (2006), who reported that reduction of *Fusarium* activity by irradiation of barley prior to steeping led to an increased growth of aerobic bacteria and yeasts during malting. Increased wort viscosity detected in the samples of irradiated barley indicated the stimulated production of high molecular weight microbial polysaccharides during malting by these species. Shifting of the balance between the microbial communities should be taken into account when modifications in the process are made, since microbe-free space in the kernel will not remain during malting.

Barley grain is an active, living matrix and changes in its metabolism have consequences for its surrounding environment and microbial communities. This study indicated that the microenvironments inside the grain tissues may differ from the ambient, and that modifications in the malting ecosystem may lead to an increase in unexpected microbial groups. H_2O_2 treatment led to enhanced multiplication of LAB, although normally H_2O_2 is considered as an antimicrobial agent against LAB (Paper I, Figure 1). This treatment was used as a reference treatment in Paper I, in which we studied the mechanisms and role of microbes in grain germination. H_2O_2 is an active oxygen source and can directly diffuse into plant tissue and promote faster germination (Wilhelmson *et al.* 2006). Improved seed vigour and enhanced production of CO_2 by the barley grain was observed after H_2O_2 treatment, and increased CO_2 concentration inside the husk layers was the most probable cause for the enhanced growth of LAB (Paper I). Morris and Monier (2003) reported that diffusion of molecules such as gases throughout the biofilms in plant tissues depends on the nature of exopolymeric substances. The gel-like nature of the polymeric matrix and its degree of hydration may reduce the diffusion of oxygen, nutrients and other

substances in plant biofilms. Previous studies have also reported that an increase in the aeration or in the oxygen supply in steeping resulted in a higher multiplication rate of LAB (Leino *et al.* 1994, van Campenhout *et al*. 1999). In agreement with our results, van Campenhout *et al.* (1999) reported that rather than the enhanced oxygen supply, the concomitant increase of CO_2 production by more vigorous germinating barley explained the higher counts of LAB.

This study clearly indicated that malt properties could be tailored by modifying the microbial activity during malting. However, it also highlighted the importance of monitoring the changes in both bacterial and fungal communities when changes are made in the malting ecosystem.

4.2 Molecular approaches for the characterization of microbial communities in the malting ecosystem (Papers I, III)

Knowledge of microbial dynamics during malting has been limited, partly because the conventional approaches often resulted in an incomplete picture of the true microbial diversity present. Recently-developed molecular approaches provided us with new tools to assess the microbial communities in the malting ecosystem. In the present study, molecular PCR-based approaches were applied to study the complexity of microbial communities in the malting ecosystem in addition to the traditional cultivation methods.

In order to apply the molecular techniques in malting ecosystem research, an efficient, rapid and simple DNA extraction method for grain samples was required. The extraction of total microbial community DNA was the first step in the ecosystem analysis. However, DNA extraction from the mixed microbial cultures was challenging, because it was difficult to extract DNA from all species with the same efficiency. Some bacteria and fungi were very difficult to lyse due to their very complex cell walls and capsules. Therefore, the proportion of these microbes in a population might have been underestimated. The extraction method had to be suitable for mycelia, fungal spores as well as for encapsulating microbes in order to obtain the total picture of microbes present in the grain ecosystem. The DNA extraction method described in Paper I was effective for processed grain samples as well as for mycelia and spores of fusaria

and slime-forming microbes such as *Leuconostoc* and *Rhodotorula,* whereas several DNA extraction protocols tested gave poor yields for barley and malt grains and did not extract the DNA from the indicator strains (Figure 9).

Table 8. DNA yield and purity of the barley and malt samples. The ratio between the readings at 260 nm and 280 nm provided an estimate of the purity of the DNA. Pure preparations of DNA have an $OD_{260/280}$ value of 1.8. Results are averages of duplicate samples.

	FastDNA kit		**Plant kit with mechanical lysis**		**Alkaline detergent extraction**	
	Barley	Malt	Barley	Malt	Barley	Malt
DNA, µg/ml	172	257	82	54	44	46
$OD_{260/280}$	1.82	1.82	1.7	1.84	1.29	1.39

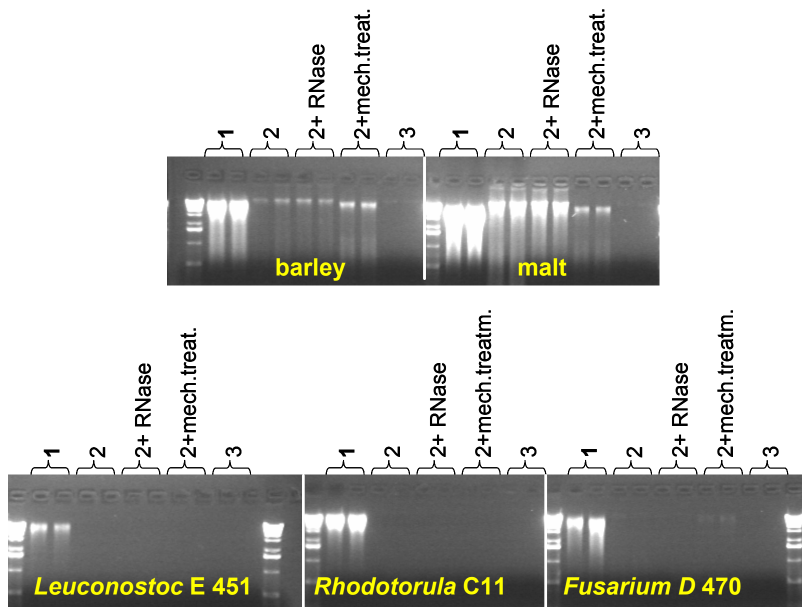

Figure 9. Extraction of total genomic DNA from barley, malt and pure cultures. 1. FastDNA Spin Kit for Soil, 2. NucleoSpin® Plant DNA -kit without mechanical treatment, with RNase treatment (2+RNase), and with CTAB procedure combined with mechanical treatment (2+mech.tretm), 3) Extraction with an alkaline detergent. Microbial pure cultures: Leuconostoc citreum E451, Rhodotorula glutinis C11 and Fusarium graminearum D470.

The highest yield of pure microbial DNA was obtained with a commercial kit developed for soil samples (Table 8). Purity is an important factor because residues from grain matrix may act as inhibitors in the PCR reaction. Grains contain many different components such as phenolic compounds, polysaccharides and proteins which may disturb the PCR-analysis of barley and malt samples. The protocols developed for soil research seemed to be suitable for barley and malting ecosystem studies. Soils represent probably the most complex microbial environments, since several thousands of microbial species can be detected in a single soil sample in addition to other organic matter (Amann *et al.* 1995). Sarlin *et al.* (2006) successfully applied the same approach for the quantification of trichothecene-producing *Fusarium* species in barley and malt with real-time PCR.

We also developed a simple and fast DNA extraction protocol for yeast pure cultures isolated from a malting ecosystem (Paper III). Some yeast cells associated with barley were extremely difficult to disrupt due to their very complex cell walls and capsules. Therefore, DNA was extracted from young cultures (18–24 h) with a DNA-kit, which combined both enzymatic and mechanical lysis.

PCR-DGGE was demonstrated to be a useful tool for monitoring microbial population dynamics in the malting ecosystem. In the present study, PCR-DGGE was applied to explore the bacterial dynamics after antibacterial treatments with universal bacterial primers targeted to the variable region V6–V8 of the bacterial 16S rRNA gene. The sample specific DNA-fingerprints clearly revealed the changes in the individual bacterial populations after the antimicrobial treatments (Paper I: Figure 2 and Table II). Furthermore, PCR-DGGE profiling combined to the partial sequencing of selected 16S rRNA gene fragments revealed that unidentified bacterial species were detected in the malting ecosystem. We showed that Agrobacterium spp. and some other previously uncultured Gram-positive bacteria belonged to the predominant bacterial community of barley and most probably multiplied during the malting process. The role of these bacteria remains to be solved. Culture-independent molecular techniques such as PCR-DGGE applied to monitor microbial diversity in various types of food and beverage fermentations have revealed microbial populations and microbial interactions not detected by plating techniques (Giraffa & Neviani 2001, Ercolini 2004).

Interestingly, barley DNA gave a strong signal in the PCR-DGGE analysis with universal bacterial primers. However, the strong band given by the barley DNA

was clearly differentiated in the gel from the bacterial bands (Paper I, Figure 2). Lopez *et al.* (2003) and also Normander and Prosser (2000) reported that universal bacterial primers can amplify plant chloroplast rDNA. It is obvious that amplification of non-target organisms can limit the detection of true bacterial or fungal species, because the DNA from non-target organisms competes with the bacterial DNA for primers and deoxynucleoside triphosphates during PCR amplifications. To overcome this problem, primers can be targeted to specific microbial groups and thus it is possible to monitor the presence, succession and persistence of certain microbial populations within the complex community.

4.3 Indigenous Gram-negative bacteria of barley influence grain germination and wort separation (Paper I)

Steeping was a critical step in malting with respect to microbial activity, and the procedures carried out during the first hours of malting greatly influenced the quality of the final product. We showed that suppression of the Gram-negative bacteria during the steeping phase was advantageous with respect to both grain germination and malt quality (Papers I, II, IV). Even a 2 log reduction of aerobic bacterial counts, mainly consisting of the genera *Erwinia*, *Enterobacter, Pantoa, Pseudomonas* and *Rahnella*, led to an improved controllability of grain germination and accelerated wort separation. In addition, higher (0.5–0.8%) extract content of worts was obtained after suppression of Gram-negative bacterial communities (Paper I: Table III, IV and Figure 6).

The present study revealed that limiting bacterial growth, especially that of Gram-negative bacteria during the first steep, improved the germination measured as rootlet growth, whereas limiting the fungal community did not affect the germination (Paper I, Table III). Figure 10 illustrates the improved grain vitality after suppression of bacterial growth. Our study was in agreement with previous investigations indicating that there is a strong interaction between microbial community and grain germination, even for non-dormant and good quality malting barley (Doran & Briggs 1993, Gaber & Roberts 1969, Kelly & Briggs 1992, van Campenhout *et al.* 1998, 1999, van Campenhout 2000). Kelly and Briggs (1992) reported that the reduction of overall microbial load on barley stimulated the barley metabolism.

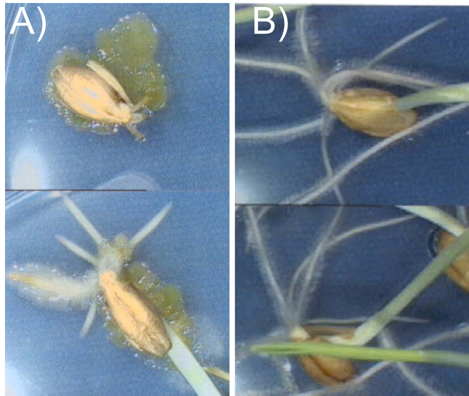

Figure 10. Enhancement of grain vitality by restricting the bacterial community. Barley grains were steeped in water (A) or in water containing antibacterial antibiotics (B). Grains were then germinated on agar plates.

It has been proposed that microbes, especially aerobic bacteria, inhibit grain germination by competing with grain tissue for oxygen (Doran & Briggs 1993). Dormant barley grains have been especially vulnerable to microbial competition. We showed that in the case of normal mature barley the improved grain germination was not related to oxygen availability (Paper I, Figures 4 and 5). Our results are supported by the studies of van Campenhout *et al.* (1998, 1999), who reported that the existence of microbial respiratory activity does not necessarily mean that there is actually competition between plant tissues and microbes. We suggested that the complex microbial communities inside the barley layers form a physical barrier and that reducing bacterial loads improved the root emergence. As early as in 1944, Bishop suggested that bacteria developing at the time of harvest form a slime or mucus covering the grain embryo, and thereby inhibit grain germination. This theory was also supported by the results of van Campenhout *et al.* (1998) who reinfected barley with *Pantoea agglomerans* after an antimicrobial treatment and detected reverse effects such as inhibited water uptake and shorter acrospires.

This study revealed that by suppressing Gram-negative bacteria during malting, wort separation performance was accelerated even when normal high quality malting barley was used (Paper I: Figure 6, Table IV). Mash filterability is a complex process influenced by many different factors related to barley components. The filtration rate is dependent on the complexes formed between proteins and pentosans, β-glucans, residual starch and lipids (Muts & Pesman 1986).

The improved wort filtration (Figure 11) could be due to the reduction of slime-forming bacteria. Anderson (1993) reported that microbes may secrete flocculents onto the surface of malt, thus affecting the porosity of the filter bed. Our previous studies revealed that severe mash filtration difficulties observed in the presence of split barley kernels were caused by an intensive growth of exopolysaccharide-producing bacteria such as *Pseudomomas* species (Haikara & Home 1991, Laitila *et al*. 1999). It has been shown that even small amounts of bacterial polysaccharides had a negative impact on wort separation (Kreisz *et al.* 2001). Furthermore, non-viable malt-derived bacteria, particularly those of submicron size, have been shown to disturb both wort and beer separation and to cause visible hazes in the final product (Walker *et al.* 1997). Amongst the bacteria identified were *P. agglomerans*, *Erwinia* spp., *Micrococcus* spp. and *Bacillus* spp. The release of bacteria and their exopolysaccharides from grain matrix during mashing depends on the agitation. Therefore, differences in the malt behaviour would be expected in different breweries due to the various types of industrial practices. Wort separation is often the rate-limiting step in the brewhouse and poor run-offs cause production losses (Andrews 2006, Stenholm *et al.* 1996). Good and balanced filterability of malt is a prerequisite for an effective brewing process.

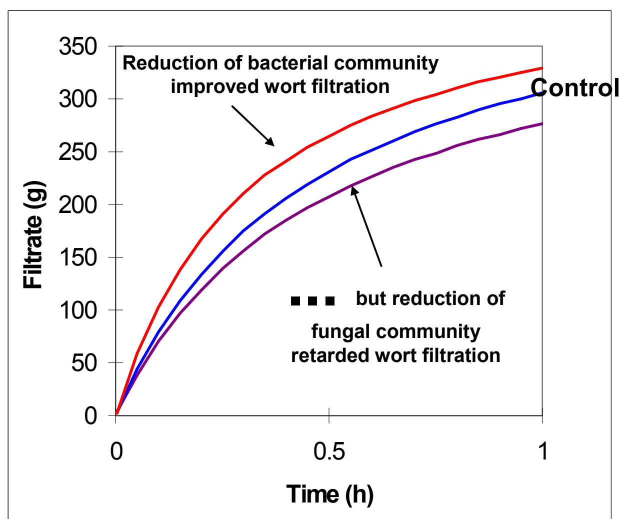

Figure 11. Effects of bacterial and fungal communities on wort filtration.

4.4 Fungal communities contribute to the production of hydrolytic enzymes (Paper I)

The good filtration rates obtained after suppressing the bacterial communities could also be due to the enhanced fungal activity. We showed adverse effects on wort separation performance when fungal communities were suppressed (Figure 11). Fungal communities, especially filamentous fungi, have been shown to be significant sources of cell-wall degrading enzymes such as β-glucanases and xylanases (Angelino & Bol 1990, Bol & Huis in't Veld 1988, Hoy *et al.* 1981, Sarlin *et al.* 2005, Yin *et al.* 1989). We measured approximately 20% lower xylanase and microbial β-glucanase activities in the malt samples after addition of antifungal antibiotics into the steeping water (Paper I, Table V). The antifungal treatments clearly reduced the growth of common filamentous fungi representing the genera of *Fusarium, Alternaria, Dreachslera* and *Mucor*. The enzyme production of some general filamentous fungi associated with the malting ecosystem was also verified using minimal medium containing 0.5–1% β-glucan and xylan (Table 9). As seen from Table 9, several strains, especially fusaria, intensively degraded β-glucan and xylan. Van Campenhout (2000) estimated that approximately 2/3 of malt xylanase activity was derived from the microbial communities and only 1/3 from the grains. Filamentous fungi also contributed to production of amylolytic enzymes (Table 9).

This study also gave clear indication of fungal involvement in the proteolysis (Paper I, Table IV). Antimicrobial treatments with antifungal antibiotics effectively suppressed the *Fusarium* fungi and led to restricted proteolysis which was recorded as decreased levels of soluble and free amino nitrogen (FAN) in worts. (Paper I: Table I and IV). In accordance with the present results, significant decrease of wort colour and FAN levels were recorded after reduction of Fusarium activity by irradiation of barley prior to malting (Kottapalli *et al.* 2006). Several studies have indicated that heavy infection of barley with *Fusarium*-fungi inthe field conditions or during malting is linked to increased proteolytic activity (Haikara 1983, Sarlin *et al.* 2005, Schwarz *et al.* 2001, 2002).

Table 9. Production of extracellular enzymes by filamentous fungi isolated from barley.

Fungal species	**Strain code**	**Enzyme activities**		
		Amylase	**β-glucanase**	**Xylanase**
Achremonium polychronum	D-96653	+	+	+
Alternaria alternate	D-76024	+	+++	+++
Aspergillus ochraceus	D-00808	+	++	+
Cochliobulus sativus	D-76039	+	+++	+++
Fusarium avenaceum	D-80141	+	+++	++
F. cerealis	D-96601	+	++	++
F. culmorum	D-80148	+	+++	+++
F. equiseti	D-82087	+	+++	++
F. graminearium	D-82169	+	+++	+++
F. graminearium	D-95470	+	+	+++
F. langsethiae	D-03931	+	++	++
F. oxysporum	D-80134	+	+++	+++
F. poae	D-76038	+	++	+++
F. sambucinum	D-77056	(+)	+++	++
F. sporotrichioides	D-82175	+	+++	+++
F. sporotrichioides	D-72014	-	++	++
F. tricinctum	D-96607	+	+	+++
Penicillium verrucosum	D-01847	+	+++	+
Pyrenophora teres	D-89395	+	+++	+++

- negative result: no degradation
(+) weak production
+…++ positive result: a clear zone
+++ intensive degradation of the substrate

4.5 Lactic acid bacteria (LAB) as a tool for management of microbial communities during malting and for enhancement of malt processability (Paper II)

Paper I indicated that malt processability can be further improved even in the case of high quality material, especially by suppressing the Gram-negative bacteria. In the present study L. plantarum E76 and P. pentosaceus E390 cultures were added to the steeping water of normal malting barley in order to balance the microbial communities and enhance malt processability. The malting trials were carried out in 25 kg pilot scale with five different two-row barley varieties.

4.5.1 LAB treatments alter steeping conditions

The inoculation stage and the composition of the starter preparation are critical with respect to the functions of microbial cultures in bioprocesses. Due to the rapid activation of the indigenous microbial communities at steeping (Papers I–IV), the starter cultures were added in this stage. It has been shown that whole LAB cultures (cells and spent medium) are needed for maximal antimicrobial action, because the antimicrobial effect of LAB is to a large extent based on the compounds present in the culture broth, and the growth medium also provides beneficial nutrients for the starter strains (Haikara *et al.* 1993, Laitila *et al.* 2002, Niku-Paavola *et al*. 1999). In order to investigate the effects of spent medium and chemical acidification on the malting performance, we prepared unfermented MRS without glucose and supplemented it with 2.5% lactic acid (MRS-LA).

Biological and chemical acidification notably changed the environment around the kernels in steeping and thus influenced grain physiology. The present study revealed that low pH during steeping (Paper II, Table 2) resulted in delayed grain germination and reduced water uptake. Delayed germination was recorded as decreased carbon dioxide production during the first air rest and as reduced rootlet growth. However, after the third day of malting 91–98% of the kernels were germinated in all the samples. After the steeping period, the moisture content of barley was approximately 1% lower in the treated samples compared to the control samples. Therefore, it was necessary to spray extra water on the LAB or MRS-LA samples in order to obtain the desired moisture level of 46%. Despite the delays in germination during the first days of malting, malt

modification and enzyme production were not disturbed. In this study low steeping and germination temperatures were applied for all the samples. The deficiencies in grain germination could have been compensated by temperature and respiratory control during processing. In accordance with the present study, van Campenhout (2000) reported that starter performance could be improved by respiratory control of barley after the inoculation stage. This study highlights the importance of monitoring and controlling the whole ecosystem when starter technology is applied.

4.5.2 LAB treatments suppress bacteria and *Fusarium*-fungi

LAB starter cultures proved to be an effective way of balancing the bacterial communities in malting. A statistically significant ($P < 0.001$) 2–3 log reduction in the number of aerobic bacteria was recorded after addition of LAB cultures (Paper II: Figure 2). Pseudomonads were particularly sensitive to LAB treatments (Figure 12). As shown in Paper I, this group was linked to impaired wort separation performance. The antibacterial action of LAB was partly due to the organic lactic acid and low pH, as similar effects were obtained with chemically acidified MRS. In addition to organic acids, *L. plantarum* E76 is known to produce low molecular weight antimicrobial compounds (Niku-Paavola *et al*. 1999).

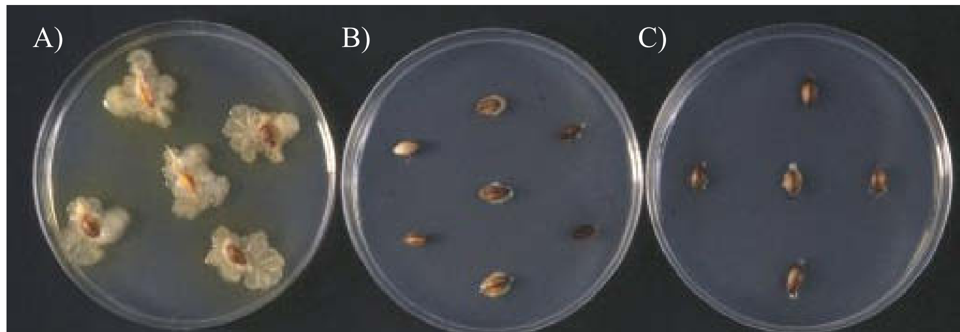

Figure 12. Inhibition of slime-forming Pseudomonads by lactic acid starter cultures. Barley grains were steeped in water (A) or in water containing P. pentosaceus E390 (B) or L. plantarum E76 starter culture (C). Steeped grains were placed on Pseudomonas-selective agar.

In addition to Gram-negative bacteria, LAB treatments changed the composition of the indigenous LAB populations, mainly comprised of *Leuconostoc* bacteria. *Leuconostoc* are often present in high numbers in the early stages of malting (Booysen *et al*. 2002, O'Sullivan *et al.* 1999, van Waesberghe 1991). However, they are relatively sensitive to acidic conditions, and it has been shown that lactic acid contributes to the early elimination of Leuconostocs in plant fermentations (Harris 1998). In addition to Gram-negative bacteria, Leuconostocs are capable of producing slimy microbial polysaccharides, which may cause severe filtration problems (Haikara & Home 1991). Therefore the suppression of this group is advantageous with respect to mash filterability.

LAB treatment also restricted the growth of *Fusarium* fungi (Paper II, Figure 2). The antifungal potential of *L. plantarum* E76 and *P. pentosaceus* E390 against *Fusarium* moulds has been demonstrated in several laboratory scale experiments (Haikara *et al.* 1993, Laitila *et al.* 1997, 1999, 2002). This pilot scale study with five different barley varieties supported the previous findings. The antifungal action of LAB is often due to several interrelated mechanisms, and it can be partly explained by the production of organic acids. The strongest antimicrobial potential was obtained with *L. plantarum* E76. Several *L. plantarum* strains are known to produce specific antifungal compounds, which are involved in antifungal actions (Karunaratne *et al.* 1990, Gourama & Bullerman 1995, Lavermicocca *et al.* 2000, Magnusson & Schnürer 2001, Magnusson *et al*. 2003, Niku-Paavola *et al.* 1999, Sjögren *et al.* 2003, Ström *et al.* 2002, Valerio *et al*. 2004).

In the present study the growth of fusaria was also restricted by chemical acidification of the steeping water with MRS-LA. We previously reported that *Fusarium* species differed in their susceptibility to LAB antimicrobials and that the growth of *F. avenaceum* was suppressed with lactic acid, whereas *F. culmorum* and *F. graminearum* fungi were not influenced by lactic acid and low pH (Laitila *et al.* 2002). On the contrary, we have observed that small amounts of lactic acid even improved the growth of *F. culmorum* (our unpublished data). Restriction of fusaria with the chemical acidification could be explained by the presence of the sensitive *F. avenaceum* species, which was the most commonly detected *Fusarium* species in Finnish grain samples in recent years (Yli-Mattila *et al.* 2002). However, *Fusarium* diversity differs in different crops and locations. Therefore, organic acids alone are not recommended for the control of fusaria.

L. plantarum E76 and *P. pentosaceus* E390 have also shown antimicrobial potential in other cereal-based bioprocesses. Katina *et al.* (2002) successfully utilized these strains in wheat sourdough breads, in which they notably inhibited rope spoilage caused by *Bacillus* species. Furthermore, a combined culture of E76 and E390 effectively suppressed the growth of clostridia during the storage of brewer's spent grains (Suomalainen *et al.* 1995). As a thermophilic bacterium *P. pentosaceus* E390 survived even in spent grains coming directly from the brewery silo. This study also showed that the E390 strain survived better than E76 in the kilning process and could be recommended in applications at high temperatures. The different characteristics of the two strains make them applicable to different types of bioprocesses.

4.5.3 LAB treatments enhance malt processability

In the pilot scale study carried out with five different barley samples, the mash filterability in the control samples was considered to be good but still the starter treatments could improve the filtration rates and volumes (Paper II, Table 3, Figure 4). The beneficial effects were more pronounced with *L. plantarum* E76 than with *P. pentosaceus* E390 or MRS-LA treatment. Acidification of the first steeping water with LAB effectively restricted Gram-negative bacteria. Furthermore, enhanced enzyme activities measured after LAB-treatment led to a more intensive degradation of barley cell-wall polysaccharides, which was noticed as decreased β-glucan level and wort viscosity. It has been shown that lowering the pH will promote the activity of hydrolytic enzymes, with the exception of α-amylase (Lewis 1998). Samples obtained after *L. plantarum* E76 treatment exhibited higher xylanase activities than chemically acidified samples, with a consequent beneficial contribution to the lautering performance. This study confirmed our previous findings that LAB treatments during steeping notably improved wort separation performance (Haikara & Laitila 1995, 2001, Laitila *et al.* 1999).

In accordance with our results, Lowe *et al.* (2005a) added LAB cultures into the steeping and found that all biologically acidified malts exhibited higher β-glucanase activities compared to the malt produced from untreated barley. They reported that the enhanced enzyme potential obtained by biological acidification could be used to compensate for reduced enzyme activities when other adjuncts

such as unmalted barley are used in the brewing process (Lowe *et al.* 2004, 2005b). Biological acidification of the malt is also an alternative for adjustment of wort pH without direct application of lactic acid, which is not permitted in some countries.

This study showed that LAB treatments and chemical acidification of the steeping water tended to enhance proteolysis, which was observed as intensified wort colour and as increased amounts of soluble nitrogenous compounds in worts. Activation of endogenous proteolytic enzymes due to the low pH could partly explain the increased protein degradation. Furthermore, LAB treatment may play a role in restricting the movement of nitrogen into roots. Lowe *et al.* (2005) reported that treatments that inhibit rootlet growth frequently caused an increase in soluble nitrogen levels, presumably because nitrogen is not drained away into the roots. Excessive levels of soluble nitrogen are undesirable as they may have a negative impact on foam and haze properties of beers and because they decrease the microbiological stability of the finished beer (Bamforth & Barclay 1993). However, higher proteolysis in malt is preferred when a larger proportion of starch adjuncts are used as a raw material.

4.6 Significance of yeasts in the malting ecosystem (Papers II, III, IV)

Paper I reported that suppression of the bacterial communities promoted yeast growth. Furthermore, our studies with lactic acid starter cultures revealed that the inoculation of LAB into steeping always promoted yeast growth and enhanced the production of microbial β-glucanase and xylanase (Paper II, Haikara *et al.* 1993, Haikara & Laitila 1995, 2001). However, the source of these microbial enzymes was largely unknown and we suggested that enhanced growth of yeast communities could partly explain the increased enzyme activities. High numbers of yeasts and yeast-like fungi have frequently been detected in the malting ecosystem (Bol & Huis in't Veld 1988, Douglas & Flannigan 1988, Flannigan *et al.* 1982, Haikara *et al.* 1977, O'Sullivan *et al.* 1999, Petters *et al.* 1988). However, surprisingly little has been known about the species dynamics in the malting ecosystem and their contribution to malt properties. Therefore, we set up a study in which the diversity of yeast and yeast-like fungi was investigated in an industrial malting ecosystem (Paper III).

4.6.1 Diversity of yeasts and yeast-like fungi

Four industrial malting runs were thoroughly investigated (Paper III). Figure 13 shows the yeast growth in industrial maltings. We also studied the effect of growth temperature on the yeast counts. As shown in Figure 13, yeasts in the malting ecosystem were capable of growing at 15 °C as well as at 25 °C. Our results were in agreement with those of Petters *et al*. (1988), who also found that the yeasts in the malting ecosystem were favoured by their ability to grow at low temperatures prevailing in steeping and germination. The malting ecosystem also harboured yeasts capable of growing at 37 °C (Figure 13). However, greater variation in the number of thermotolerant yeasts was observed within batches compared to the populations at 15 or 25 °C. These yeasts probably originated from the malting equipment, and batch to batch variation in the process environment and in malting procedures could explain the observed fluctuation. It has been shown that a specific microbial community develops in each malting plant and it also has significant effects on the properties the final product (O'Sullivan *et al*. 1999, Petters *et al.* 1988).

As seen from Figure 13, kilning appeared to have little effect on the viable yeast counts. Only tenfold reduction in yeast counts was observed during kilning. In fact the first hours of kilning before the temperature breakthrough, especially in the top layers of the grain bed, appeared to be rather favourable for yeast growth. Under normal environmental conditions, the vegetative yeast cells are rapidly inactivated by temperatures of 60–65 °C. (Fleet 1992.) This study revealed that a large proportion of the yeast community was composed of encapsulating yeasts, which could explain the high number of survivors in the kilned malt. In the malting ecosystem the microbial cells embedded in thick biofilms were well protected. Schwarz *et al.* (1995) also reported a large increase in the ergosterol content during the early hours of kilning, indicating that fungal growth (both yeasts and filamentous fungi) was accelerated. It is clear that a significant amount of fungal metabolites such as enzymes is formed during this stage, which may later have an impact during the mashing stage. In addition, synthesis of harmful fungal metabolites such as mycotoxins has been reported during kilning (Schwarz *et al*. 1995). Therefore, kilning can also be regarded as an important step with respect to microbiological safety.

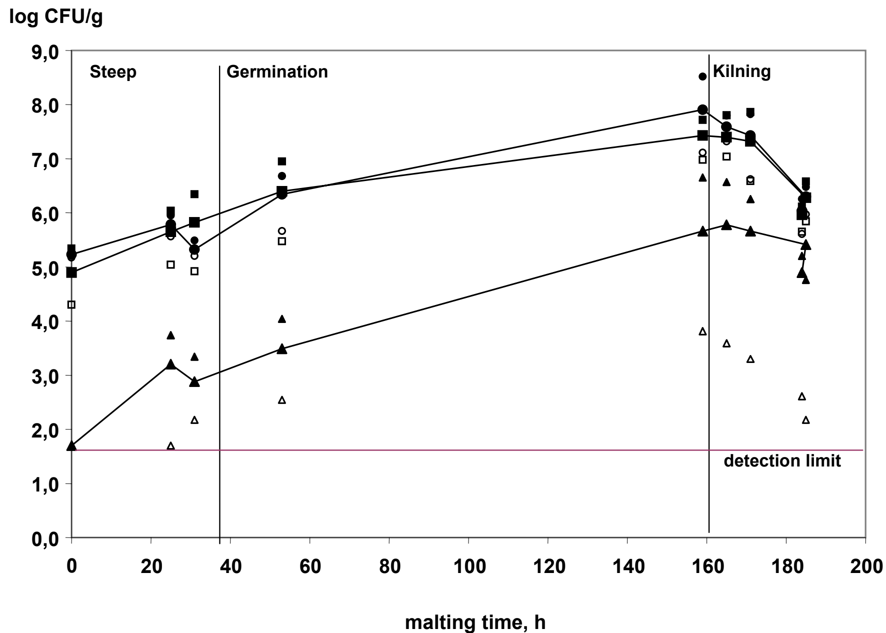

Figure 13. Growth of yeasts during the industrial scale malting process. •) Yeasts cultivated at 15 °C, ■) yeasts cultivated at 25 °C and ▲) yeasts cultivated at 37 °C. Open small symbols are the minimum values and closed small symbols are the maximum values detected in determinations.

The main goal of this study (Paper III) was to obtain an overall picture of the yeasts in the industrial malting ecosystem. A total of 136 malting samples were collected from four industrial processes. More than 700 yeast isolates were first discriminated with PCR-fingerprinting using an oligonucleotide primer (M13) targeting simple repetitive DNA sequences (microsatellites). This protocol has been widely applied in yeast typing and allows the discrimination of yeast species even at the subspecies level (Loureiro 2000). Yeasts representing different fingerprint types were then identified by sequence analysis of the D1/D2 domain of the 26S rRNA gene (Fell *et al*. 2000, Kurtzman & Robnett 1998).

We detected 25 species of ascomycetous yeasts belonging to eight genera and 18 species of basidiomycetous yeasts belonging to six genera from the malting trials with Saana barley from the 2001 crop (Table 10). Previously only 10 ascomycetous and 6 basidiomycetous yeast species have been reported from

barley and malting samples (Douglas & Flannigan 1988, Flannigan 1969, Flannigan *et al.* 1982, Flannigan & Dickie 1972, Kottheimer & Christensen 1961, Noots *et al.* 1999, Petters *et al.* 1988, Tuomi *et al.* 1995, Tuomi & Rosenqvist 1995).

Table 10. Yeast species detected in the industrial malting ecosystem. Identified strains were deposited in the VTT Culture Collection and their 26S rRNA gene sequences were deposited in GenBank under the numbers shown in Tables 2 and 3 in Paper III.

Genera	Species
Ascomycetous	
Candida	*anglica, cylindracea, fermentati, intermedia, natalensis, pararugosa, picinguabensis, saitoana, sake, silvae, solani, Candida* sp. I and II [1]
Clavispora	*lusitaniae*
Galactomyces	*geotrichum*
Geotrichum	*silvicola*
Hanseniaspora	*clermontiae/meyri* [2], *uvarum*
Issatchenkia	*orientalis*
Pichia	*anomala, fabianii, fermentans, guilliermondii*
Saccharomyces	*exiguus*
Williopsis	*californica*
Yeast-like fungi	
Aureobasidium	*pullulans*
Exophiala	*dermatidis*
Basidiomycetous	
Bulleromyces	*albus*
Cryptococcus	*albidosimilis, curvatus, hungaricus, macerans, magnus, victoriae, wieringae, Cryptococcus* sp. I, II, III and IV [3]
Filobasidium	*globisporum*
Rhodotorula	*glutinis, pinicola*
Sporobolomyces	*roseus, ruberrimus*
Trichosporon	*brassicae*

[1] Two sets of *Candida* isolates did not match closely enough to any sequences present at the time in the database.

[2] Species cannot be separated by D1/D2 sequencing.

[3] Four groups of undescribed *Cryptococcus* species, indicated as *Cryptococcus* sp. I–IV, were found on the basis of D1/D2 sequences (Paper III, Table 3).

All the identified yeast species were detected at least at a level of 10^4–10^5 cfu/g. Some minor species may have been overlooked in the present study and thus the yeast diversity in the malting ecosystem could be even greater. Basidiomycetous yeasts dominated the yeast community of barley (Paper III, Table 4). Furthermore, they were frequently detected during the first days of malting. The growth of basidiomycota was favoured by the low temperatures during steeping. Many basidiomycetous species have temperature optima below 20 °C (Deak 1991). In contrast to basidiomycetous species, ascomycetous yeasts dominated at the end of germination and during the first hours of kilning. We found 20 different ascomycetous yeasts in the samples taken after 5 h of kilning, whereas only five basidiomycetous yeasts were detected in the same samples (Paper III, Table 4). The occurrence of ascomycetous yeasts was obviously due to their ability to grow better at the higher temperatures than basidiomycetous yeasts.

This study provided a clear indication of the vast yeast diversity in the malting ecosystem. It is obvious that even more yeast heterogeneity could be expected due to the differences between barley crops as well as between industrial practices in different locations. Even some potentially novel species were found in the malting ecosystem. The unidentified isolates have been subjected to further characterization. To confirm that the strains represent different species, multigene sequence analysis is required (Kurtzman & Robnett 2003). Analysis of combined gene sequences such as internal transcribed spacer regions of the rRNA genes (ITS), the actin gene and mitochondrially encoded genes will provide more information of the genetic relationships than partial analysis of the 26S rRNA gene (Daniel & Meyer 2003, Fell et al. 2000, Kurtzman and Robnett 2003).

4.6.2 Production of hydrolytic enzymes

Yeasts and yeast-like fungi isolated from the malting ecosystem were screened for the production of amylase, β-glucanase, cellulase and xylanase (Table 11). We showed that several yeasts, especially basidiomycetous species, were active producers of various types of enzymes with a potentially positive contribution to malt processability.

The enzyme production was determined using minimal medium containing 0.5–1% of a specific substrate as sole carbon source. Some enzyme activities,

especially those of ascomycetous yeasts, may have been underestimated in the plate-screening in which a complex polysaccharide was the only carbon source. Strauss *et al.* (2001) reported that some ascomycetous yeasts showed cellulase activity in the presence of glucose. In the malting ecosystem complex interactions with the other organisms such as filamentous fungi and bacteria as well as with the germinating grain influence the growth and activity of the yeast community. Horn (1984) reported that growth of *Pichia guilliermondii* associated with corn was considerably increased in the presence of amylolytic filamentous fungi. The yeast community may also be a source of proteolytic and lipolytic activities. Although these enzymes have not been as extensively studied as polysaccharide-hydrolysing enzymes, they are also known to influence malt and beer quality.

Table 11. Yeasts and yeast-like fungi derived from an industrial malting ecosystem and showing production of extracellular enzymes in the plate-screening assay.

Amylase	β-glucanase	Cellulase	Xylanase
A. pullulans	*A. pullulans*	*A. pullulans*	*A. pullulans*
B. albus	*B. albus*	*B. albus*	*B. albus*
C. natalensis	*C. albidosimilis*	*C. macerans*	*C. albidosimilis*
C. albidosimilis	*C. curvatus*	*C. magnus*	*C. magnus*
C. macerans	*C. macerans*	*C. wieringae*	*C. victoriae*
C. wieringae	*C. magnus*	*Cryptococcus sp.* I, II, III	*C. wieringae*
Cryptococcus sp. I, II	*Cryptococcus sp.* III, IV	*E. dermatidis*	*Cryptococcus sp.* I, II, III and IV
C. hungaricus	*E. dermatidis*	*F. globisporum*	*E. dermatidis*
S. roseus	*F. globisporum*	*G. silvicola*	
S. ruberrimus	*R. pinicola*		

Malt-derived yeasts could be a source of enzymes with specific characteristics applicable for cereal bioprocesses. Several cold-adapted yeasts, also found in this study, produce enzymes which are of interest to the food industry. For example the application of cold-active pectinases from *Cryptococcus* species has attracted considerable interest in the fruit and vegetable processing industry (Birgisson *et al.* 2003).

4.6.3 Antifungal potential of yeasts derived from the malting ecosystem

Our study also revealed that yeasts derived from the malting ecosystem had a antifungal potential (Paper IV). *In vitro* screening with the plate assay indicated that several ascomycetous strains belonging to the species *A. pullulans, C. sake, C. saitoana, G. geotrichum*, *P. anomala* and *P. guilliermondii* showed antifungal activity against field and storage fungi (Paper IV, Table 1).

Table 12. Antifungal potential of selected yeasts and yeast-like fungi against Fusarium-fungi in a plate-screening assay. - No inhibition, + suppression of mould growth.

	VTT-D	*Aureobasidium pullulans* D-04**1014**	*Candida sake* C-9**5520**	*C. saitoana* C-04**524**	*G. silvicola* D-04**559**	*Pichia anomala* C-04**564**	*P. anomala* C-04**565**	*P.guilliermondii* C-04**568**	*Cryptococcus albidus* C-92**012**	*Cr. albidosimilis* C-04**508**	*Cr. curvatus* C-04**536**	*Cr. magnus* C-04**540**	*Rhodotorula pinicola* C-04**571**
F. avenaceum	80**141**	+	+	+	+	+	+	+	+	+	+	+	+
F. cerealis	96**601**	+	+	+	+	+	+	+	-	+	+	-	-
F. culmorum	80**148**	-	-	+	+	+	+	+	-	-	-	-	-
F. equiseti	82**087**	-	+	+	+	-	+	-	-	-	-	-	-
F. graminearum	82**169**	-	-	+	+	+	+	+	-	-	-	-	-
F. graminearum	95**470**	+	-	+	+	+	+	+	-	+	-	-	-
F. langsethiae	03**931**	-	-	+	+	+	+	-	-	-	-	-	-
F. oxysporum	80**134**	-	-	-	+	+	+	-	-	-	-	-	-
F. poae	76**038**	-	-	+	-	+	+	+	-	-	-	-	-
F. sambucinum	77**056**	-	-	+	+	+	+	-	-	-	-	-	-
F. sporotrichioides	82**175**	-	-	-	+	+	+	+	-	-	-	-	-
F. sporotrichioides	72**014**	+	-	+	+	+	+	-	+	-	-	-	-
F. tricinctum	96**607**	-	-	-	+	+	+	-	-	-	-	-	-

The main emphasis was on the suppression of *Fusarium* growth. All the yeast strains tested could prevent the overgrowth of *F. avenaceum* D141 in the plate-screening assay, whereas *F. oxysporum* D134 and *F. tricinctum* D607 strains were restricted only by *G. silvicola* D559 and *P. anomala* strains C564 and C565 (Table 12). *P. anomala* C565 strain was selected for malting experiments in order to verify the antifungal potential of malt-derived yeast in malting with naturally infested barley.

Our results are supported by previous investigations which also reported the antifungal activity of these yeasts in other applications (Fredlund *et al.* 2004, Passoth *et al.* 2005, Saligkarias *et al*. 2002, Schena *et al.* 2003, Wisniewski *et al.* 1991). Several biocontrol yeasts are nowadays commercially available. For example yeast strains belonging to *Candida oleophila*, *Cryptococcus albidus*, and *Metschnikowia fructicola* are commercialized and have been successfully applied to prevent pre- and post-harvest fungal diseases of fruits and vegetables (Boekhout & Robert 2003, Janisiewicz & Korsten 2002). *P. anomala* J121 strain has been applied to control the spoilage moulds during storage of high moisture feed grains (Druvefors *et al.* 2002, Passoth *et al.* 2005). *Geotrichum candidum*, also known as an IFBM malting yeast, has been developed for inhibiting fungal growth and mycotoxin production in malting (Boivin & Malanda 1997, Boivin 2002).

The effects of one potential biocontrol agent, *P. anomala* C565, were also examined in malting with naturally contaminated barley exhibiting gushing potential. To our knowledge this is the first report that shows the effects of *P. anomala* against fusaria in malting and the consequent effect on the overall malt quality. *P. anomala* occurs naturally in cereals and is classified as safe (biosafety level 1 microorganisms) (Druvefors 2004). This study revealed that the addition of *P. anomala* C565 (isolated from an industrial malting process) into the steeping was highly suppressive to *Fusarium* and *Mucor*-fungi (Figure 14). We showed that *P. anomala* inhibited the production of fungal hydrophobic proteins during malting and prevented gushing (Paper IV, Figure 3, Table 2). Hydrophobins are among the most important structural proteins found in filamentous fungi (Ebbole 1997). Hydrophobins are produced in response to changes in the environment and are linked to the attachment of fungi to plant surfaces (Wessels 1997). Fungal hydrophobins also act as gushing inducers of beer, although the production of gushing factors in malting is still largely an unknown phenomenon

(Haikara *et al*. 2000, Sarlin *et al.* 2005). It is well known that intensive *Fusarium* growth is a part of the normal malting process. However, to our knowledge the production of gushing factors occurs only rarely in industrial malting processes. The results of the present study indicate that some suppression of *Fusarium* growth and hydrophobin production probably occurs in normal industrial practice with the aid of the indigenous yeasts community.

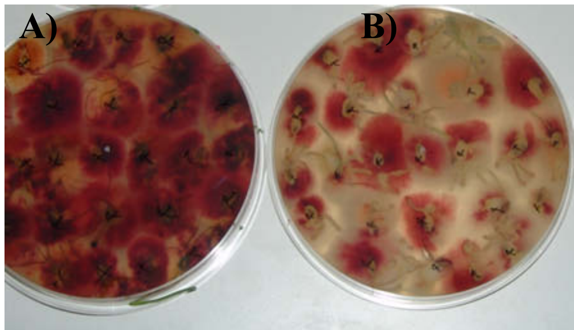

Figure 14. Fusarium growth restriction by P. anomala C565 added to the steeping water. Kernels contaminated with Fusarium fungi after steeping on CZID plates. Control (A), P. anomala C565 (B) (IV).

Although the mode of action of the antifungal activity remains to be revealed, the results indicated that *P. anomala* C565 competed for space with fusaria. As a fast growing organism, *P. anomala* colonized the outer layers of barley and suppressed the adherence of fungal contaminants to the barley surface during steeping. Antifungal action of antagonistic yeasts is often due to several mechanisms and hitherto no single mechanism has been shown to be responsible for the complete antimicrobial action. The mechanisms are poorly understood, especially in such complex ecosystems. Competition for nutrients and space has often been suggested as the main mode of action. In addition, the antifungal action of antagonistic yeasts includes induction of the plant defence system, production of lytic enzymes such as β-1-3 glucanase and chitinase, which degrade the fungal cell wall, or secretion of antimicrobial compounds such as killer proteins (Janisiewicz & Korsten 2002, Masih & Paul 2002, Passoth & Schnürer 2003). Druvefors *et al.* (2002) suggested that the antifungal effect of *P. anomala* was due to the synergistic action of ethyl acetate and ethanol produced by *Pichia* in oxygen-limited environment. This study revealed that ethyl acetate was indeed detected in the gaseous atmosphere of the malting drum in *P. anomala*-treated samples, which might partly explain the antifungal action against fusaria.

P. anomala C565 had no significant effect on the bacterial communities or on grain germination. We showed that *P. anomala* utilized the grain volatile metabolites as a substrate for growth, without disturbing the grain's normal germination process (Paper IV, Table 3, Figure 5). Furthermore, the final malts were well modified. However, *P. anomala* C565 treatment tended to retard mash filterability when added to the steeping water. As a strongly antagonistic organism *P. anomala* suppressed the growth of other yeasts and filamentous fungi and led to a decreased production of microbial β-glucanase and xylanase, which could partly explain the reduced filtration rate (Paper IV, Table 4). Furthermore, the slight increase in wort viscosity in *Pichia*-containing samples indicated the presence of high-molecular weight polysaccharides. Kreisz *et al.* (2001) reported that malt-derived yeast polysaccharides such as mannan and glycogen may have a significant impact on the haze levels of filtered beer. Therefore, precautions must be taken when selecting biocontrol agents to malting applications. The negative impact of *P. anomala* on filtration performance may limit its use in malting applications alone. In the present study a rather high inoculum level was used (10^6 yeast cells / gram of barley) in steeping. The inoculum level and stage in the malting process need further investigations. However, this study clearly confirmed the previous findings (Druvefors *et al.* 2002, Fredlund *et al.* 2004, Passot & Schnürer 2003, Petersson & Schnürer 1998, Petersson *et al.* 1999) that *P. anomala* strains have great antifungal potential and can be used in cereal-based processes to inhibit the growth of spoilage fungi.

4.7 Tailoring malt properties with combined techniques (Paper IV)

In order to improve the retarded wort filtration, *P. anomala* C565 was combined with LAB (IV). *L. plantarum* E76 addition to the first steeping water led to enhanced xylanase and microbial β-glucanase activities even when combined with an antagonistic yeast (Paper IV, Table 5). Furthermore, *L. plantarum* E76 restricted the growth of aerobic bacteria, especially pseudomonads (Paper IV, Figure 7). Thus, the negative impact of *P. anomala* C565 on wort separation performance could be counteracted (Paper IV, Figure 6).

The preliminary characterisation using the FESEM microscope supported our results that the extensive slime formation observed in the control samples (Figure

15A) could be suppressed with LAB and yeast cultures (Figure 15B), although further studies are needed to verify these results. FESEM pictures also clearly visualized the complex nature of microbial communities within the malting process. Control of this ecosystem with one single microbial species is hardly possible.

This study clearly showed that combining of LAB with an antagonistic yeast was advantageous. The combination of two or more different well-characterized microbes derived from the malting ecosystem offers a possibility to use their different properties, thus making the system more robust. In a complex malting ecosystem the diverse microbial communities consisting of bacteria, yeasts and filamentous fungi must be taken into account together with barley activity. Combining mixed cultures with other alternatives, such as heat treatment of barley or modified atmosphere treatment, could result in a successful control strategy for malting purposes.

Some of the expected benefits related to the balancing of microbial communities in the malting ecosystem include:

- ensured product safety
- uniform process throughout the year and crop (balancing seasonal variations)
- accelerated brewhouse performance
- improved extract yield
- novel types of functional ingredients with a natural and healthy image.

The microbiota management during malting cannot replace strict control of the incoming barley. However, the well-characterized microbial mixtures consisting of barley and malt-derived bacteria and fungi offer an additional measure to guarantee microbiological safety particularly in years when poor weather conditions favour the growth of toxigenic and gushing-active fusaria. Furthermore, this study clearly revealed that processability of the malt produced from high-quality barley without any expected problems could be enhanced by modifying the microbial community during malting. A modified malting process is an alternative to increase the functional properties of barley malt. Microbes contribute to the production of technologically and nutritionally valuable substances such as enzymes, organic and phenolic acids and vitamins. Microbes also open up several possibilities to design specific flavours and to develop new malted ingredients.

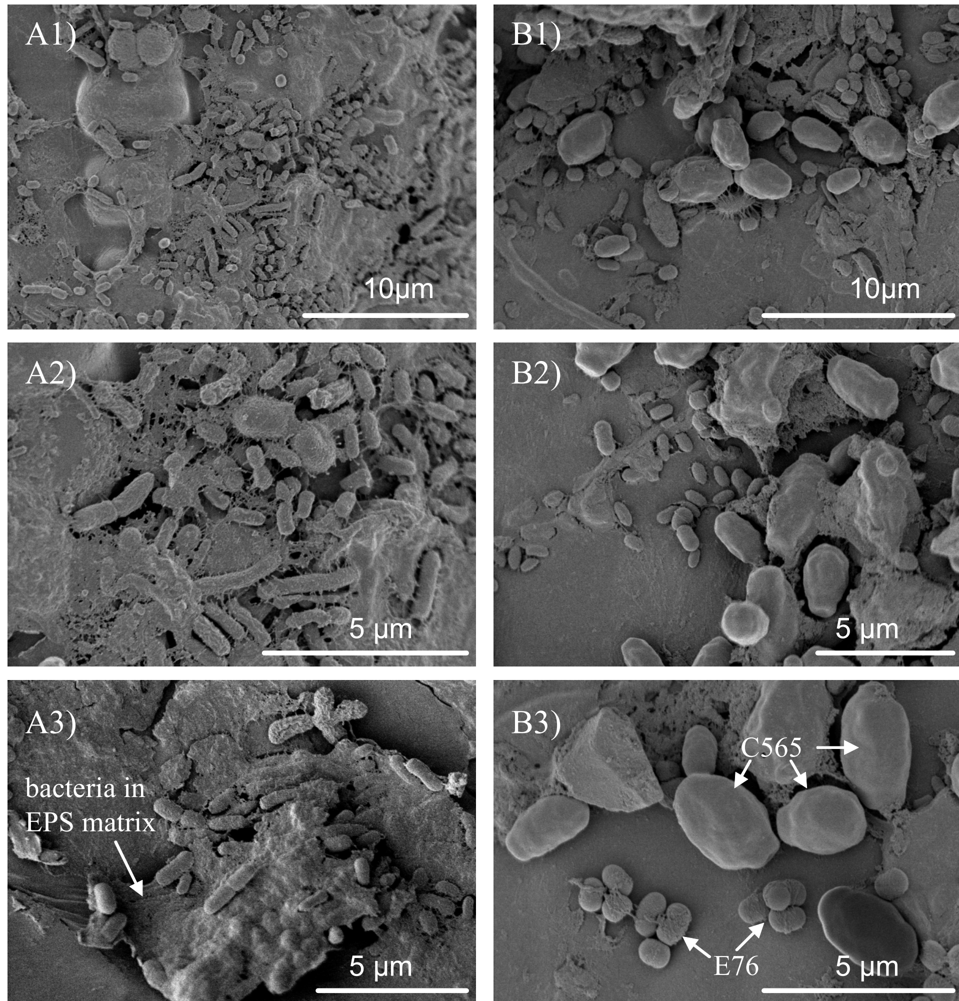

Figure 15. FESEM micrographs of the steeped grains show 2 day old biofilms in the outer layers of barley (between the testa and the outer epidermis). Control samples (A) show extensive slime formation (EPS matrix) around the indigenous microbial cells in the grain layers, whereas less exopolymeric matrix was observed in the starter-treated samples (B). Samples were derived from the malting experiment in which L. plantarum E76 (E76) and P. anomala C565 (C565) were added to the steeping waters (Paper IV). Control samples were steeped in water. FESEM pictures Mari Raulio, University of Helsinki & Arja Laitila, VTT.

5. Conclusions

This study showed that the malting ecosystem is indeed a dynamic process and exhibits continuous change. The dynamics of the microbial communities in the malting ecosystem were influenced by the initial microbial load, interactions between microbial populations during processing, the process conditions and selective operations such as addition of starter cultures or antimicrobials. Furthermore, it was recognised that each process step could be a source for additional microbes. Improved understanding of the complex microbial communities and their role in malting enables a more controlled process management and the production of high quality malt with tailored properties.

The main findings of this thesis work were:

- The microbial communities consisting of various types of bacteria, yeasts and filamentous fungi formed tight networks in the outer layers of barley and were well protected in the grain biofilms. Steeping of barley in water induced rapid microbial growth and intensive production of exopolymeric substances within the husk tissues. Furthermore, inhibition of one population within the complex ecosystem led to an increase of non-suppressed populations, which should be taken into account, because the shift in microbial community dynamics may be undesirable. Both bacterial and fungal communities should be monitored simultaneously when changes are made in the process.

- Traditional, culture-dependent approaches underestimated the microbial species diversity. With the aid of the new powerful molecular tools we showed that the diversity of microbes in the malting ecosystem was greater than expected. Even previously undescribed bacterial and yeast species were found in the malting ecosystem. Some of the new microbial groups may be considered as barley/malting ecotypes, meaning that they only exist in the barley-malt chain.

- The microbial growth and activity during the first hours of steeping greatly influenced the grain germination and the quality of the final malt. Both bacteria and fungi including filamentous fungi and yeasts had

significant impacts on the malting performance. Therefore, steeping can be regarded as the critical step in malting process. Kilning had surprisingly little effect on the viable counts of microbes. In fact the first hours of kilning were even rather favourable for microbial activity. Therefore, kilning can also be regarded as an important step with regard to the microbiological safety of malt.

- Suppression of Gram-negative bacteria during steeping was advantageous with respect to grain germination and wort separation. In addition, more extract was obtained after suppression of the bacterial communities.

- The fungal communities, both filamentous fungi and also yeasts, were significant producers of β-glucanase and xylanase contributing to wort filtration and extract yield. Filamentous fungi, especially fusaria, were also involved in the proteolysis.

- Biological acidification of the steeping water with *Lactobacillus plantarum* VTT E-78076 (E76) and *Pediococcus pentosaceus* VTT E-90390 (E390) enhanced the malt processability. The starter cultures promoted yeast growth and restricted the growth of slime-forming bacteria and fusaria. Enhanced enzyme activities and lower wort viscosity and β-glucan content were associated with the application of LAB in malting, leading to notably improved filtration performance.

- A numerous and diverse yeast community, consisting of a wide variety of ascomycetous and basidiomycetous species, was an important part of the industrial malting ecosystem. The most frequently isolated ascomycetous yeasts belonged to the genera *Candida, Clavispora, Galactomyces, Hanseniaspora, Issatchenkia, Pichia, Saccharomyces* and *Williopsis* and the basidiomycetous yeasts to *Bulleromyces, Filobasidium, Cryptococcus, Rhodotorula, Sporobolomyces* and *Trichosporon*.

- Many of the yeasts associated with barley and malting, especially the basidiomycetous yeasts, produced enzymes degrading plant cell walls with a potentially positive contribution to the malt enzyme spectrum.

- Several ascomycetous yeast strains showed antagonistic activities against field and storage fungi *in vitro* using a plate-screening assay. The most potent strains were *Candida saitoana* VTT C-04524, *Geotrichum silvicola* VTT D-04559, *Pichia anomala* VTT C-04565 and *P. guilliermondii* VTT C-04568.

- *Fusarium* growth during malting and the production of fungal hydrophobic proteins, also known as gushing factors, was suppressed with *P. anomala* C565, which is a naturally occurring yeast in the malting ecosystem.

- *P. anomala* C565 possesed antifungal activity, but unfortunately had a negative on the wort filtration performance, which may limit its use in malting applications alone. The filtration performance was recovered by combining *L. plantarum* E76 and *P. anomala* C565.

- Combining lactic acid bacteria with fungal cultures enhances the usefulness of starter technology in complex cereal ecosystems such as malting.

6. Future outlook

Well-characterized microbes derived from the malting ecosystem offer a natural tool for achieving safe and balanced microbial communities when added to a malting ecosystem in a controlled way. Furthermore, by modifying the microbial communities during malting, the brewing efficiency of malt could be notably improved. Multifunctional starter mixtures also offer a tool to produce novel ingredients with desired nutrional and technological properties for the food and beverage industry. Tailor-made malted cereals could be used in the baking industry and also in the production of malt-based non-alcoholic beverages and new types of functional products. However, the transfer of knowledge obtained from laboratory and pilot scale experiments into real complex industrial processes requires further investigations. Scaling up is a critical step in the development of a controlled and economically profitable industrial process.

In addition, the formulation of microbial cultures and inoculation procedures needs to be optimized. In the present study, LAB and yeast cultures were cultivated separately in commercial laboratory media. However, for large-scale applications their costs are prohibitive. In addition, MRS-medium contains constituents, such as components of bovine origin, not approved in food production. Recently, we developed a cereal-based alternative medium for the production of protective cultures in which the expensive and unsuitable components were replaced with a food-grade malt-sprout extract (Laitila *et al*. 2004). The cereal-based medium supported the growth of LAB at the same level as the commercial MRS. In addition, enhanced antimicrobial activity against Gram-negative bacteria and fusaria was observed. The cereal-based media can be used as a low-cost alternative to MRS for producing high cell yields and good antimicrobial activity.

PCR-DGGE was demonstrated to be a useful tool for monitoring population dynamics in the malting ecosystem. In the present study only the universal bacterial primers were used to evaluate the changes in the bacterial community after various antimicrobial treatments. Our further studies have been directed to using group-specific bacterial and fungal primers in order to profile the changes in the specific microbial groups in barley in field conditions as well as in industrial malting ecosystems.

The basis of the malting ecosystem research is that both microbial and grain activities are monitored and controlled simultaneously. The recent developments in molecular biology, especially in the field of gene expression, have opened up new possibilities. Multiplexed and quantitative analysis of gene expression is an attractive approach, because it would enable the simultaneous monitoring of grain and microbial activities from the same sample. Recently microarrays and chips allowing multiplexed DNA or RNA analysis have become widely used tools in research. An alternative approach based on affinity capture was developed at VTT (Rautio *et al.* 2006, Satokari *et al*. 2005, Söderlund *et al.* 2001). The TRAC (transcript analysis with the aid of affinity capture) approach enables monitoring the expression of a few or several dozens of genes simultaneously. Transcriptional profiling in the malting ecosystem would then enable early detection of barley metabolite synthesis, and at the same time the synthesis of various microbial metabolites such as mycotoxin synthesis could be monitored in a single assay.

In-depth knowledge of the microbes and their activities as well as early detection of changes are crucial in the entire barley-malt-beer chain from field to consumer with respect to product and process safety. The multitarget control strategies in combination with novel monitoring technologies will open up new possibilities for the improvement of process efficiency and also for product innovations.

As shown here, microbes are an integral component of the barley malt. Although this thesis only touched upon the relationship between barley and the diverse microbial communities associated with the grains, it clearly showed that microbes have great potential when properly controlled. Microbes can be considered as nature's own tiny tailors.

Like Friedrich von Flotow aptly wrote in his opera Martha (Lemoine & Marchand 1999):

"Long live malt! Long live hops! They are the salt of life!"

Microbes have the power to make this salt mixture of life much more interesting.

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PAPER I

Indigenous microbial community of barley greatly influences grain germination and malt quality

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Indigenous Microbial Community of Barley Greatly Influences Grain Germination and Malt Quality

Arja Laitila[1,4], Erja Kotaviita[2], Petri Peltola[3], Silja Home[1] and Annika Wilhelmson[1]

ABSTRACT

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The present study was carried out to investigate the impacts of bacterial and fungal communities on grain germination and on the malting properties of good-quality two-row barley. In order to suppress the growth of bacterial and/or fungal communities, various antibiotics were added to the first steeping water of barley. This study was also designed to explore the dynamics of the bacterial community in the malting process after antimicrobial treatments by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). The diverse microbial community played an active role in the malting ecosystem. Even previously undescribed bacterial species were found in the malting ecosystem. Suppression of the bacterial community mainly consisting of Gram-negative bacteria was advantageous with respect to grain germination and wort separation. In addition, more extract was obtained after antibacterial treatments. The fungal community significantly contributed to the production of microbial β-glucanases and xylanases, and was also involved in proteolysis. An improved understanding of the complex microbial community and its role in malting enables a more controlled process management and the production of high quality malt with tailored properties.

Key words: Bacteria, barley, fungi, malt quality, malting, PCR-DGGE.

INTRODUCTION

Malting is a complex biological process in which the germination of barley leads to the synthesis of hydrolytic enzymes and degradation of the grain structure. Malt modification, *i.e.* the degradation of barley endosperm cell walls and to a limited extent of the cell content, is the main objective in malting. In addition to germinating barley, the malting process involves another metabolically active component: the diverse microbial community that naturally colonizes the grains. The indigenous community harbours a wide range of microbes including numerous species of Gram-negative and -positive bacteria, yeasts and filamentous fungi[17,20,32,41,46]. In addition, each process step can be a source of additional microbes and their metabolites[32,45]. Conditions that enable grain germination also favour microbial growth. Imbibed water rapidly activates the dormant microbes present in barley and therefore steeping can be regarded as a crucial step in malting with respect to microbial activity[32,41,45]. Microbial activity remains high during germination. Finally, the removal of water during kilning halts both grain and microbial activities. However, kilning has little effect on the viable counts of microbes, which are generally higher in the finished malt than that in the native barley[31,32,46]. The kilning regime has been identified as a significant factor in controlling the microbial community[52]. In summary, the malting process can be considered as a complex ecosystem consisting of germinating grain and a diverse microbial community. Whenever the malting process is studied or changed, both components should be considered.

The microbial community actively interacts with the barley grains. Microbes produce metabolites including plant stimulating hormones which enhance grain germination[53]. They also contribute to the grain enzyme activity by being important producers of amylolytic, proteolytic and cell wall-degrading enzymes with potentially positive effects on malt characteristics[16,17,26,41,47,50,51,62]. In addition, malt-derived bacteria and fungi with known and carefully selected characteristics can be applied as starter cultures and they offer natural ways to improve the safety and processability of malt[4,31,35].

The microbial community may also have negative impacts, leading to variability in malt quality and in the worst cases causing severe process failures. The activity of barley microbes is linked to several unwanted phenomena such as interference with barley respiration, reduction of grain viability, secretion of toxic compounds (i.e. mycotoxins) or gushing factors inducing overfoaming of bottled beer, production of off-odours and -flavours, formation of components causing premature yeast flocculation and production of extracellular substances causing filtration problems later in the brewing process as well as visible hazes and turbidity of beer[11,13,22,27,41,48,59].

Microflora management in the whole barley to beer chain is important with respect to both process and product safety and quality. Early detection and identification of changes in the microbial community is an important component of quality control. However, knowledge of the dynamics of the microbial community during the malting

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process is still limited, partly because the traditional culture-based isolation and identification approaches are laborious and time-consuming and often result in an incomplete picture of the true microbial diversity present. In recent years, there has been a trend towards culture-independent approaches. New powerful analytical tools enable us to investigate complex microbial ecosystems in their natural environment. Direct DNA/RNA extraction approaches from environmental samples, coupled with polymerase chain reaction amplification and community profiling techniques have become widely applied in studying microbial ecology in complex environments[12,38]. Denaturing gradient gel electrophoresis (DGGE) is perhaps the most commonly used culture-independent fingerprinting technique for studying the response of community dynamics to environmental variations. This technique has also demonstrated its potential in food-related ecosystems[12,19] and has been applied in beverage fields such as whisky[54,55] and wine fermentations[34]. PCR-DGGE detects the predominant species of a community without discriminating living from dead cells or cells in a non-culturable state. The main populations, constituting 90–99% of the total community, are displayed in the profiles[38]. Advantages and disadvantages of the PCR-DGGE technique were reviewed by Ercolini[12] and by Muyzer[39].

Although it has been generally accepted that microbes have an important role in the production of high quality malt, more knowledge is still needed on the impacts of specific microbial populations within the complex ecological situation. Due to several unwanted properties, such as production of mycotoxins, fungi and especially filamentous fungi have attracted more attention during the recent years, whereas less attention has been paid to the bacterial community and its effects on grain germination and malt quality. The aim of the present study was to investigate the impacts of the indigenous microbial community present in good-quality barley on grain germination and on malt properties. The growth of bacteria and/or fungi was suppressed at the beginning of steeping by adding various antimicrobial mixtures in the first steep water. In addition to a traditional culture-based approach the culture-independent PCR-DGGE technique was applied to study the complexity and behaviour of bacterial communities in malting ecosystems.

MATERIALS AND METHODS

Barley analyses

Barley samples were analyzed using the following Analytica-EBC[14] recommended methods: moisture content (EBC 3.2), protein content (EBC 3.3.1), germination capacity (EBC 3.5.2) and germination energy (EBC 3.6.2). Before malting, barley samples were sieved to remove grains <2.5 mm.

Malting experiments

Barley (*Hordeum vulgare* L, Poaceae, cultivar Scarlett, crop 2004) samples (300 g) were malted in a specially designed, computer controlled micro-malting equipment with a separate drum for each sample (Hulo Engineering, Helsinki, Finland). Triplicate malting experiments were carried out. All barley samples were steeped in 2 L of water or in water containing antimicrobials at 18°C for 8 h, followed by a 16-h air rest (20°C) and a second steep (2 h, 18°C). Antimicrobials or hydrogen peroxide (H_2O_2) were added to the first steep water. The moisture content of grains was measured daily and kept constant (46–47%) by adding water. The barley was then allowed to germinate for 5 days at 16°C and dried (kilned) in warm air (4 h 50°C, 3 h ramp to 60°C, 2.5 h 60°C, 3 h ramp to 85°C, 1 h 85°C) in a separate kiln. The rootlets were removed before analyses.

Antimicrobial treatments were selected based on their direct effects on microbial activity. In order to restrict the bacterial community (Antibac-treatment), the first steep water was supplemented with 100 ppm chloramphenicol (Sigma, St. Louis, MO, USA) and 100 ppm chlortetracycline (Sigma). Both bacterial and fungal communities were suppressed with a mixture of the following antibiotics (Antimix-treatment): 100 ppm amphotericin B (Sigma), 400 ppm nystatin (Sigma), 800 ppm penicillin-G (Sigma), 400 ppm polymyxin B sulphate (Merck, Darmstadt, Germany) and 800 ppm streptomycin sulphate (Sigma). The bacterial and fungal antibiotics included in the mixture were also studied separately. Antifung-treatment contained 100 ppm amphotericin B and 400 ppm nystatin, whereas Bacmix-treatment contained 800 ppm penicillin, 400 ppm polymyxin B and 800 ppm streptomycin sulphate. The antimicrobial treatments were compared to samples that were treated with 0.05% hydrogen peroxide (H_2O_2) in the first steep water. H_2O_2 is generally known to improve the germination of dormant barley.

Monitoring of grain germination

The concentration of ethanol in the head space of each malting drum was analyzed continuously using a Fourier Transform Infrared Spectroscopy (FTIR) multicomponent gas analyzer Gasmet® (Temet Instruments ltd, Helsinki, Finland) with a heated, flow-through, 5 m path length sample cell. Steep water samples were taken after the first and second steep for pH analysis.

The number of germinated grains was counted daily from a sample of about 150–200 kernels until the germination rate exceeded 90%. A kernel was defined as germinated when the root was visible. Samples for analysis of alcohol dehydrogenase (ADH) activity were taken after the first steep (8 h), after the air rest (8 h steep + 16 h air rest) and after one day of germination (8 h steep + 16 h air rest + 2 h steep + 24 h germination). The ADH activity was analysed using a method described by Crawford[10], modified for barley embryos as in Wilhelmson *et al.*[61]. Samples for α-amylase activity were taken after 2, 3 and 5 days of germination. These samples were freeze-dried and the rootlets were removed. The grain was ground in a disc mill (Bühler Miag, Braunschweig, Germany) using a 0.2 mm gap between the discs. The α-amylase activity was analyzed with a Ceralpha kit (Megazyme Co., Wicklow, Ireland) using an extraction time of 30 min and assay conditions as specified by the manufacturer.

Culturing of microbial groups

Samples for the microbiological analyses were taken from untreated barley and from barley after steeping, ger-

mination and kilning (after rootlet removal). The following microbial groups were analyzed from homogenized barley samples: aerobic heterotrophic bacteria, *Pseudomonas* spp., lactic acid bacteria, and yeasts. A sample of 10 g was homogenized for 10 min with 90 mL of sterile saline in a Stomacher Lab Blender 400 (Seward Medical, London, UK). Aerobic heterotrophic bacteria were determined on plate count agar (PCA, Difco Laboratories, Detroit, USA) and *Pseudomonas* spp. on C-F-C agar (Oxoid Ltd., Basingstoke, Hampshire, UK). Samples were incubated in aerobic conditions at 30°C for 2–3 days. The number of LAB was determined on MRS agar (Oxoid) and samples were incubated in anaerobic conditions at 30°C for 5 days. To prevent fungal overgrowth of bacterial determinations, 0.001% cycloheximide (Sigma Chemical, St. Louis, MO, USA) was added to PCA, C-F-C and MRS media. Yeast counts were determined on YM agar (Difco Laboratories). Samples were incubated in aerobic conditions at 25°C for 3–5 days. Chlortetracycline and chloramphenicol (both at 0.01%) were added to YM medium to prevent bacterial growth. In addition, 0.02% of Triton-X 100 (BDH) was used to limit the spreading of fungal colonies on YM-agar. The bacteria and yeast results are expressed as colony forming units/gram barley (cfu/g).

For *Fusarium* analyses, 100 randomly selected kernels were placed on a selective Czapek-Dox agar containing Iprodion and Dichloral (CZID-agar)[15]. The CZID plates were incubated at 25°C for 7 d. Other filamentous fungi such as *Alternaria* spp., *Cephalosporium* spp., *Cladosporium* spp., *Drechslera* spp., *Epicoccum* spp., *Mucor* and *Rhizopus* spp. were determined on wet filter paper using direct plating of 100 kernels[15]. Filter paper plates were incubated at 25°C for 21 days. Fungi were identified under a stereomicroscope on the basis of typical colony form and colour. Identification was confirmed by conidia morphology with a light microscope (magnification 400×). The results are expressed as per cent of kernels contaminated with fungi.

PCR-DGGE analysis and sequencing of amplicons

Barley and process samples (after steeping, germination and kilning) from the three malting experiments were analysed by PCR-DGGE. Genomic DNA from duplicate ground samples (0.1 g) was extracted with FastDNA® Spin Kit for Soil (Q-Biogene, Carlsbad, CA, USA) according to the manufacturer's instructions with the modification that the samples were homogenized with a FastPrep cell disrupter (FP120, QBiogene, Carlsbad, CA, USA) at 6.0 m/s for 30 s four times. DNA samples were stored at –20°C.

The PCR-DGGE protocol was modified from Mättö *et al.*[40]. Universal bacterial primers U968-f (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G*AAC GCG AAG AAC CTT A-3′), containing a GC-clamp, and U1401-r (5′-CGG TGT GTA CAA GAC CC-3′) were used to amplify the V6–V8 variable regions of the bacterial 16S rRNA gene[43]. The PCR reaction mixture (50 µL) contained 1 µL of the template DNA, 0.2 µM of both primers, 0.2 mM deoxynucleoside triphosphate mix, 3 units of Dynazyme Taq polymerase (Finnzymes, Finland), and reaction buffer with 10 mM Tris-HCl (pH 8.8), 50 mM KCl and 1.5 mM $MgCl_2$. The amplification program started with initial denaturing at 94°C for 5 min, followed by 35 cycles of denaturing at 94°C for 30 s, primer annealing at 50°C for 20 s, elongation at 72°C for 40s, and final extension at 72°C for 7 min. A reagent control in which DNA was replaced with redistilled water was included in every experiment.

DGGE was performed with a DCode™ Universal Mutation Detection System (BioRad, CA, USA). PCR products were loaded onto a 38–60% gradient of urea and formamide, and electrophoresis was carried out at a constant temperature of 60°C at 200 V for 5 min and at 85 V for 16 h. Gels were stained with SYBRGreen (Molecular Probes, the Netherlands), viewed by UV transillumination and photographed with a GelDoc2000 system (BioRad, USA). A sequence ladder of reference strains previously isolated from the malting process was included in the gels to provide an indication of the possible identities of bands. The following strains, provided by the VTT Culture Collection, were included into the marker: *Enterococcus* sp. VTT E-032303 (E2303), *Lactobacillus* sp. VTT E-032313, *Lactococcus lactis* VTT E-032313 (E-2313), *Pantoea agglomerans* (basonym *Enterobacter agglomerans*) VTT E-90398 (E398), and *Pseudomonas* sp. VTT E-90397 (E397).

Similarities between the DNA fingerprints were detected with BioNumerics software version 3.0 (Applied Maths BVBA). Clustering was performed with Pearson correlation and the unweighted pair group method with arithmetic mean (UPGMA)

Distinct bands (totally 40), representing unknown organisms, were excised from the polyacrylamide gels with a sterile scalpel blade and mixed with 36 µL of sterile water and crushed with a pipette tip. Samples were incubated at 80°C for 1 h and eluted overnight at 4°C. The DNA samples were then stored at –20°C. Samples were reamplified, and for amplicons, DGGE was carried out as described above, with final purification using a QIAquick PCR purification kit (Qiagen, Mississauga, Ontario, Canada). The reclaimed DNA was amplified by PCR with primers U968, not containing the GC-clamp, and U1401. The ABI BigDye v3.1 Terminator Cycle Sequencing kit was used for sequencing reactions (Applied Biosystems, Foster City, CA, USA). Electrophoresis of the products was carried out in an ABI PRISM 3100 Genetic Analyser (Applied Biosystems, USA). The sequences were edited with DNAMAN software version 4.1 (Lynnon Biosoft, Quebec, Canada). The partial 16S rRNA gene sequences (containing a sequence between U968-f and U1401-r) were compared with the sequences of the GenBank DNA database by using the BLASTN algorithm.

Malt analyses

Malt samples were analyzed using the following EBC recommended methods: friability, fine/coarse extract, soluble nitrogen, free amino nitrogen, wort and malt β-glucan[14]. The filtration rate of the congress wort was determined by weighing the filtrate after 15, 30 and 60 min. The filtration time was calculated for 250 mL of wort. Malt α-amylase was analysed as described above. The β-glucanase activity was analyzed with a kit using azo-barley glucan as substrate (Megazyme, Wicklow, Ireland).

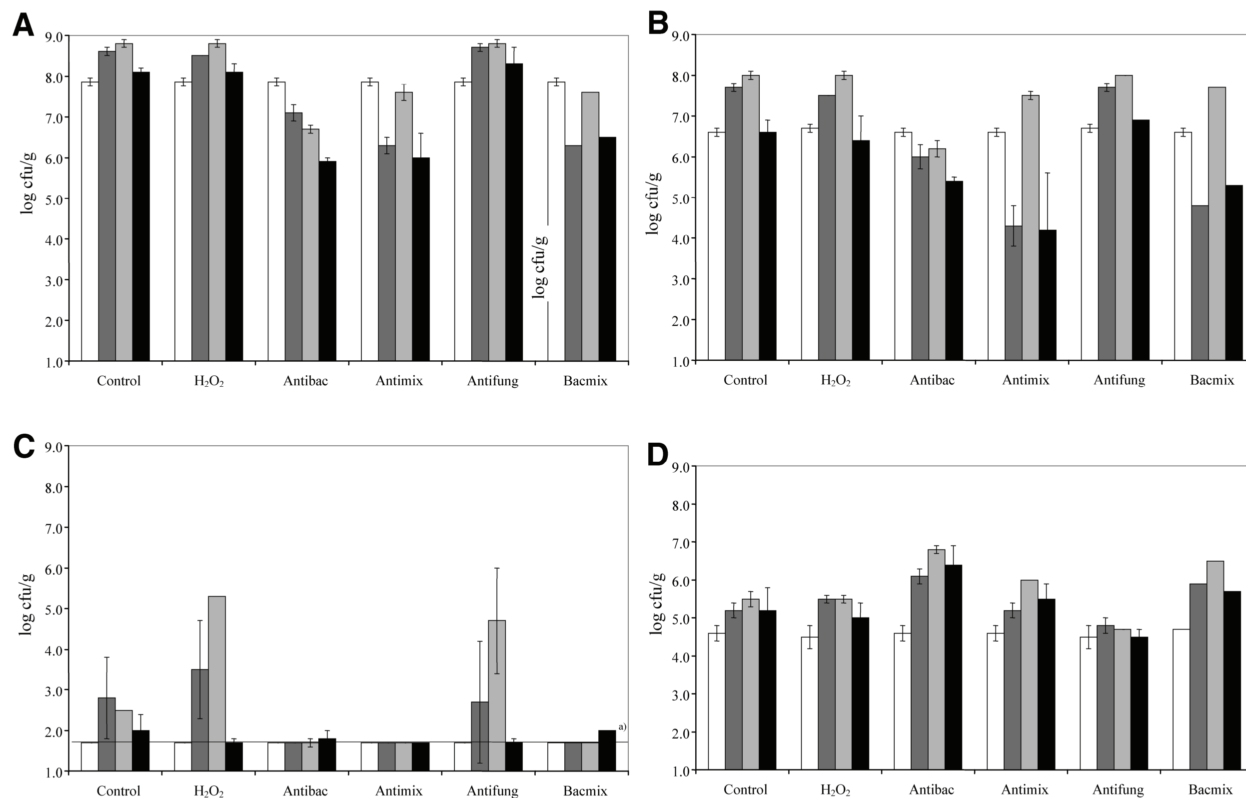


Fig. 1. The number of aerobic heterotrophic bacteria (A), *Pseudomonas* spp. (B), lactic acid bacteria (C) and yeasts (D) during malting. An antimicrobial or hydrogen peroxide (H_2O_2) was added to the first steep water. Values are means of three malting experiments, except for the Bacmix treatment which was present only in one malting.[a)] The detection limit for microbial counts was log 1.7 cfu/g. White bar = barley. Dark grey bar = steep. Light grey bar = germination. Black bar = malt.

The assay was performed at both 30 and 60°C in order to distinguish between β-glucanase of barley and microbial origin. Xylanase was analysed with an *endo*-1,4-β-xylanase assay procedure using Xylazyme AX tablets (Megazyme, Wicklow, Ireland) as substrate. Milled malt (1.00 g) was extracted in 8.0 mL of sodium acetate buffer (25 mM, pH 4.5) for 15 min at room temperature with continuous stirring (200 rpm). The flour was separated by centrifugation (1000 g). Xylanase activity was measured at 45°C. A substrate tablet was added to 0.5 mL of extract and incubated for 30 min. The reaction was stopped by adding 5.0 mL of 1% Trizma base. The absorbance was measured at 590 nm. The results are expressed as difference in absorbance between the sample and a reagent blank.

RESULTS

Changes in the microbial community due to the antimicrobial treatments

The Scarlett barley lot used in these experiments was of good malting quality with a germination capacity of 99%, a germination energy of 97%, a moisture content of 13.2% and a protein content of 10.8%. Fig. 1 shows the growth of aerobic heterotrophic bacteria (A), *Pseudomonas* species (B), lactic acid bacteria (LAB) (C) and yeasts (D) in malting after antimicrobial treatments. The bacterial antibiotics chloramphenicol and chlortetracycline were included in the Antibac-treatment, and penicillin G, polymyxin B and streptomycin sulphate in both the Antimix- and Bacmix-treatments. In addition to bacterial antibiotics, the fungal antibiotics nystatin and amphotericin B were included in the Antimix–treatments and also tested separately in the Antifung-samples. As seen from the results, H_2O_2 at the concentration studied (0.05%) had no antimicrobial effects, whereas the Antibac-, Antimix- and Bacmix-treatments led to a clear reduction in viable counts of aerobic bacteria (Fig. 1A). A decrease of 2 log units was observed during the malting process. A significant proportion of this aerobic heterotrophic bacterial population was composed of pseudomonads, and the antibacterial treatments effectively suppressed the growth of these bacteria (Fig. 1B). The number of pseudomonads was 3 × 10^6 cfu/g in the control malt samples, and 1–2 log units lower counts were measured after antibacterial treatments. However, differences were observed in antimicrobial action between the Antibac- and Bacmix-treatments (Figs. 1A and B). After addition of chloramphenicol and chlortetracycline included in Antibac-treatments, the viable number decreased gradually during malting, whereas in Antimix- and Bacmix-samples an increase of *Pseudomonas* species was observed during germination. This was also confirmed with PCR-DGGE analysis.

Only low numbers of LAB were detected in the laboratory scale malting experiments of Scarlett barley. All the antibacterial treatments effectively inhibited the growth of these gram-positive bacteria during processing (Fig. 1C),

Table I. Effects of antimicrobial treatments on the occurrence of common filamentous fungi in malting.

Fungi	Malting phase	Antibac	Antimix	Antifung	Bacmix
Fusarium	steep	0	– – –	– – –	– – –
	germination	0	– –	–	0
	malt	–	– –	–	–
Alternaria	steep	–	–	–	–
	germination	–	–	–	+
	malt	–	–	–	+
Cephalosporium	steep	–	–	+	0
	germination	–	+	+	–
	malt	+	+	+	+
Drechslera	steep	–	–	–	–
	germination	– –	– –	–	–
	malt	–	+	–	–
Mucor	steep	++	–	–	–
	germination	+++	–	–	+++
	malt	– –	– – –	– – –	–

0; no effect on fungal growth.
Reduction of fungal growth: strong ≥40% (– – –), moderate 20–40% (– –), low 1–10% (–).
Promotion of fungal growth: strong ≥40% (+ + +), moderate 20–40% (++), low 1–10% (+).

whereas suppression of the fungal community (Antifung) appeared to promote the growth of LAB during germination, although great variation was observed between the steeping and germination samples. Obviously more living space was provided for LAB growth after fungal restriction. Surprisingly, the addition of small amounts of H_2O_2 applied in steeping enhanced the LAB growth during germination (Fig. 1C). H_2O_2 treatment provided more oxygen to the grain tissue and enhanced grain germination, which was recorded as increased CO_2 production by the grain in the H_2O_2 treated samples (data not shown). A concomitant increase of partial CO_2 pressure inside the barley layers most probably stimulated the growth of these microaerophilic bacteria.

Suppression of the bacterial community with Antibac and Bacmix-treatments provided more living space for yeast growth (Fig. 1D). Approximately 10-fold higher yeast counts were detected after antibacterial treatments. The Antifung-treatment, although containing two broad-spectrum fungal antibiotics, had only little effect on the total viable counts of yeasts. However, it modified the yeast community (data not shown). The Antimix- and Antifung-treatments led to increased growth of red basidiomycetous yeasts belonging to the genera of *Rhodotorula* and *Sporobolomyces.*

The antifungal treatments clearly reduced the growth of common filamentous fungi such as *Fusarium, Alternaria, Dreachslera* and *Mucor* species (Table I). In particular, the *Fusarium*-fungi were significantly inhibited during the steeping phase and *Mucor*-fungi during kilning by the antifungal treatments. Interestingly, the reduction of the bacterial community with Antibac and Bacmix-treatments appeared to promote *Mucor* growth during germination, although lower counts were observed in the malt samples.

In addition to culturing of various microbial groups, changes in the bacterial community during malting were monitored with a direct, culture-independent approach using PCR-DGGE profiling. Fig. 2 illustrates the DGGE profiles of barley after the antimicrobial treatments. Representative bands were excised from the DGGE gels, reamplified and identified by sequencing. The number of visible bands corresponded to the number of predominant members in a bacterial community. As seen from Fig. 2, barley DNA was also amplified during the PCR reaction, although universal bacterial primers were used in this study. However, the strong band given by the barley DNA was clearly separated at the lower part of gel.

The cluster analysis of DGGE patterns revealed that samples were grouped into three main clusters, representing barley samples without antibacterial treatments (I), the samples after antibacterial treatments derived from germination and kilning (II) and the steeped samples after the antibacterial treatments (III) (Fig. 3). Barley DNA-profiles after steeping, germination and kilning contained about 10–12 distinct bands (Fig 2; lanes 1s, 1g, 1m). The Antifung-samples (lanes 4s, 4m) showed high similarity (>88%) to the control samples, except for an additional band (no 12) representing *Rahnella*/*Obesumbacterium* species which appeared in the samples taken after germination (Fig. 2, lane 4g). Antibacterial treatments were clearly recorded in the DGGE-profiles. Only 1–4 weak bands were detected in the malt samples.

Table II shows the 12 different bacterial groups identified from these experiments. Comparative sequence analysis showed that Gram-negative species dominated the bacterial community. The species with closest similarity represented mainly *Erwinia, Enterobacter, Pantoea, Pseudomonas* and *Rahnella* species. The number of LAB was low in the laboratory scale malting experiments compared to the aerobic, gram-negative bacteria (Fig. 1). Therefore, they were not displayed in the DGGE profiles (Fig. 2). Interestingly, other Gram-positive bacteria such as *Agrobacterium* spp. (band 5) and some previously uncultured bacteria (bands 7 and 10) most probably multiplied during the processing as detected by the increase of band intensity during malting.

The results of PCR-DGGE-analysis clearly revealed the changes in the individual bacterial populations. DGGE-profiling confirmed the results obtained from culturing (Fig. 1B), i.e. that the Antibac-treatment consisting of chloramphenicol and chlortetracycline was more effec-

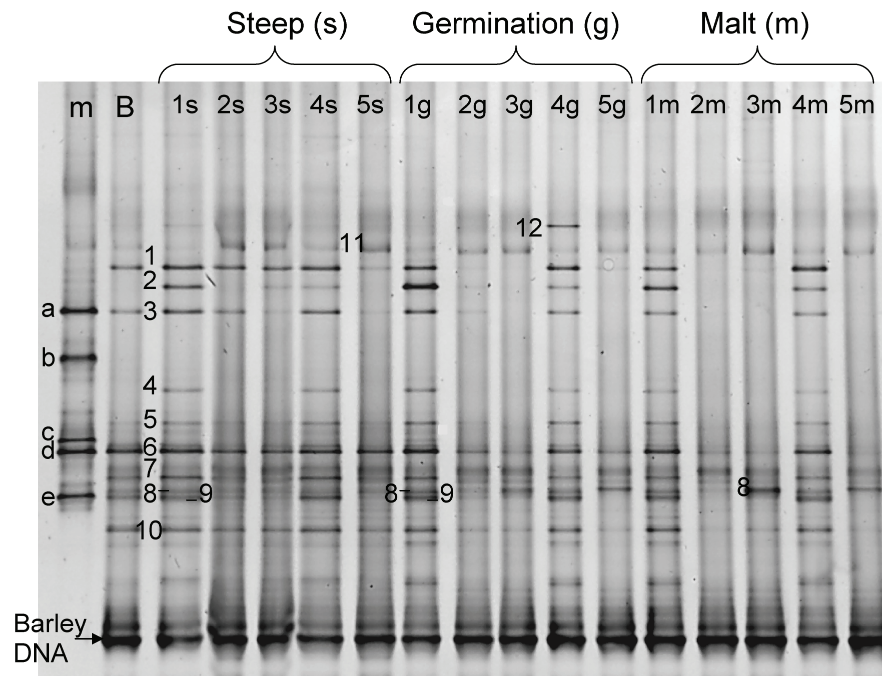


Fig. 2. 16S rRNA gene DGGE fingerprinting of bacterial communities in barley and in malting samples using the primers U968-f and U1401-r. DNA-profiles of the samples taken from native barley (B), after steeping (s), after germination (g) and after kilning (m). Different treatments are numbered as follows: 1) control, 2) Antibac, 3) Antimix, 4) Antifung, 5) Bacmix. The markers consisted of DNA fragments from pure cultures of the following strains isolated from barley and malting: a) *Pseudomonas* sp. E397, b) *L. lactis* E2313, c) *Enterococcus* sp. E2303, d) *P. agglomerans* E-398, e) *Lactobacillus* sp. E2304. The bands labelled as 1–13 are described in Table II.

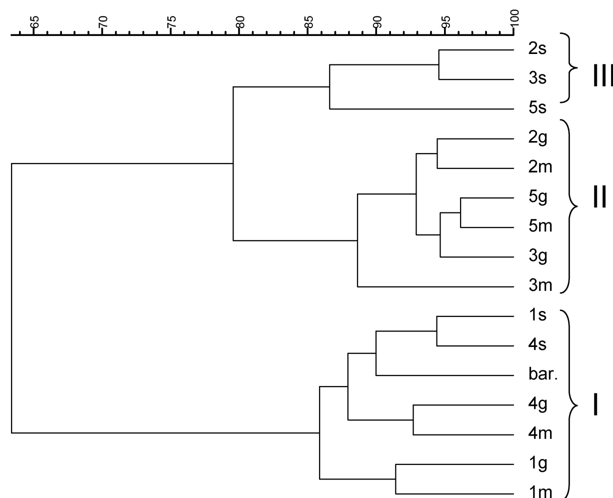


Fig. 3. Similarity index of DGGE profiles obtained from malting experiments with Scarlett-04 barley.

tive against *Pseudomonas*-species than the Antimix- and Bacmix-treatments. As seen from Fig. 2, *P. fluorescens* (band 8) was still present in the samples derived from germination (lanes 3g and 5g) and final malt (lanes 3m and 5m) after Antimix and Bacmix-treatments, whereas this species gradually decreased from the Antibac-samples (lanes 2g and 2m).

Effects of microbial community on malting performance

The normally steeped control barley germinated well in all three malting experiments (Table III). A reduction of the bacterial community with Antibac-, Bacmix-, Antimix-treatments as well as the addition of H_2O_2 further improved the germination rate of barley. The differences in germination were most noticeable after 1.3 days of malting. At that point, the antibacterial treatments had improved the germination percentage by 13–20%-units and H_2O_2 by 23%-units. The Antifung-treatment had no significant effect on germination. After three days of malting, the percentage of germinated grains was about 99% in all samples. The faster germination of the H_2O_2-treated samples resulted in a higher α-amylase activity after three days of malting, but no such effect was observed after antibacterial treatments although they also improved germination (Table III).

Ethanol was detected in the head space of the malting drum in all samples, indicating that the barley was suffering from oxygen deficiency in the beginning of malting (Fig. 4). The ethanol concentration increased during the air rest and decreased after the introduction of the second steep water. During the first day of germination, the ethanol concentration increased to about 50 ppm and then decreased almost to 0 ppm. As seen from Fig. 4A the Antibac-samples had a higher ethanol concentration than the other samples during the air rest. However, this was

Table II. Results of sequence analysis of selected DGGE bands labelled in Fig. 2.

DGGE band	Closest sequence(s) found in the GenBank database (sequence number)	Nucleotides sequenced, bp	% sequence identity
1	*Erwinia persicinus* (AJ937837.1)	401	100
2	Uncultured bacterium (AY345401.1)	357	100
	Escherichia sp. (DQ013851.1)	355	99.4
	Enterobacter sp. (AY753173.1)	355	99.4
3	*Pseudomonas* sp. (AY770691.1)	402	100
4	Uncultured bacterium (AY770937.1)	306	96.5
5	*A. tumefaciens* (AY626383.1)	403	100
	Agrobacterium sp. (DQ193597.1)	403	100
6	*Pantoea agglomerans* (DQ06572.1)	377	100
7	uncultured low G+C Gram-positive bacterium, (DQ124776.1), closest to *Paenibacillus* sp.[a]	250	93.6
8	*Pseudomonas fluorescens* (DQ146946.1)	406	100
	Pseudomonas sp. (AY486374)	406	100
	Pseudomonas synxantha (AY486374)	406	100
9	*Pseudomonas putida* (DQ 229317.1)	301	100
10	unidentified bacterium (DQ4999994.1) closest to *Curtobacterium* sp.[a]	268	92
11	*Erwinia billingae* (AM055711.1)	401	100
12	*Rahnella* sp. (DQ405247.1)	341	100
	Obesumbacterium proteus (AJ233422.1)	341	100

[a] Several bands were sequenced and an unclear sequence was obtained with forward primer. Therefore, sequencing was performed only in one direction.

Table III. The effects of antimicrobial treatments and H_2O_2 on grain germination (1.3 and 3 days of malting) and α-amylase activity (3 days of malting).

	Control[a]	H_2O_2[b]	Antibac[b]	Antimix[b]	Antifung[b]	Bacmix[b]
Δ Germinated grain, 1.3d, %	67	+23	+13 (± 2)	+19 (± 4)	–2	+20
Δ Germinated grain, 3d, %	99	0	+1 (± 1)	–1 (± 1)	–1	+1
Δ α-amylase, 3d, U/g	105	+45	+8 (± 9)	+1 (± 20)	+4	+11

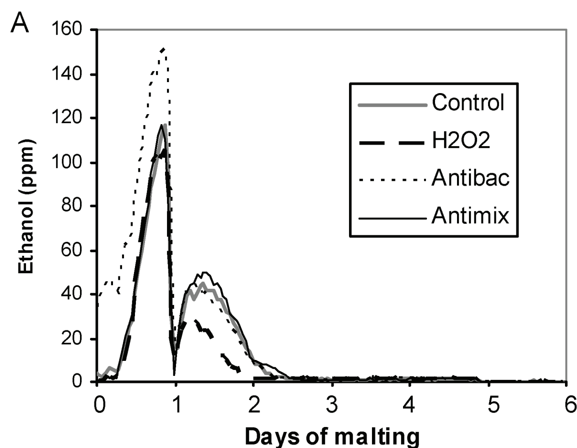
[a] The results are an average of three malting experiments.
[b] The results for treatments are expressed as difference (Δ) with respect to the control. The standard deviation of the differences (Δ) within the triplicate malting experiment is presented in brackets.

most probably an artefact, because a small amount of ethanol was needed in order to dissolve chloramphenicol in the preparation of the antibiotic mixture. Therefore it can be concluded that the antimicrobial treatments had no effect on the ethanol production profiles of barley, whereas H_2O_2 in the first steep water led to a faster disappearance of ethanol during the first day of germination, indicating a direct effect of H_2O_2 on grain physiology. The treatments had no effects on the ADH activity of the embryo during the first two days of malting. The embryo ADH activity increased slightly during the beginning of malting, and then declined (Fig. 5).

Effects of microbial community on malt quality

As seen from Tables IV and V, the antimicrobial treatments greatly influenced enzyme production and the quality of the final malt. The malt analysis results in Table IV are expressed as differences with respect to the control. In this way, the results of the three independent malting experiments can easily be compared. The malts were all well modified based on the high friability values, low wort β-glucan and extract contents. The antimicrobial treatments had no effect on the friability, but reducing the bacterial community improved the extract content by 0.5–0.8% d.w. The antibacterial treatments (Antibac and Bacmix) also increased the concentrations of soluble and free amino nitrogen by 50–90 mg/L and 16–29 mg/L, respectively. The suppression of the fungal community had an opposite effect. None of the treatments had any significant effect on the α-amylase activity or on the endogenous β-glucanase activity (30°C).

The antibacterial treatments notably improved the filterability of the congress mash, whereas the antifungal treatments had the opposite effect (Table IV, Fig. 6). Suppression of the bacterial community led to approximately 30% shorter filtration time. The bacterial community could directly influence the porosity of the mash, and reduction of the bacterial load, especially of exopolysaccharide-producing bacteria, during malting could partly explain the improved filtration performance. Furthermore, the increased enzyme activities measured from the kilned malt could also explain improved wort separation. The antifungal treatment reduced the thermostable β-glucanase activity as well as the xylanase activity (Table V), whereas the effects of the antibacterial treatments were negligible (Antibac) or positive (Bacmix). The differences



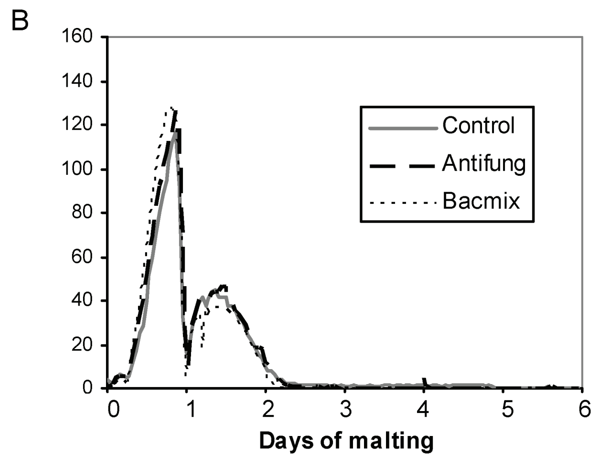


Fig. 4. Grain ethanol production after antimicrobial or H_2O_2 treatment. Ethanol concentration (ppm) was measured automatically in the head-space of the each individual malting drum. The lines represent the average values of three (Control, Antibac, Antimix), two (H_2O_2, Antifung) or one (Bacmix) malting experiment(s).

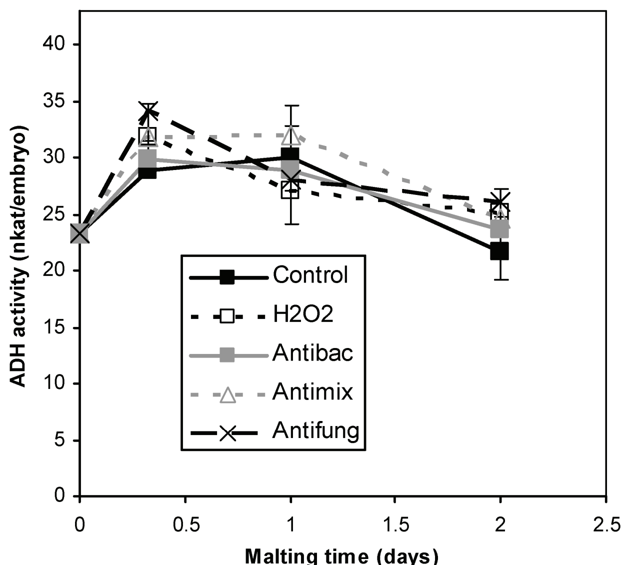


Fig. 5. The embryo ADH activity at the beginning of malting after antimicrobial or H_2O_2 treatment. Standard deviations are indicated by error bars. The figure represents one of the three malting experiments. The results of the other malting experiments were similar.

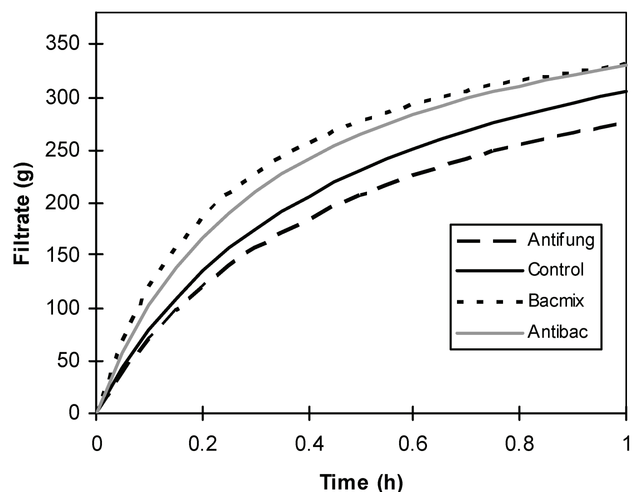


Fig. 6. Effects of antibacterial and antifungal treatments on wort separation. The figure represents one of the three malting experiments. The results of the other malting experiments were similar.

in β-glucan concentration (Table IV) were small compared to the standard deviations, but consistent with the microbial β-glucanase activities (60°C).

DISCUSSION

The present study confirmed that steeping was a critical step in malting with respect to microbial activity, and that procedures carried out during the first hours of malting had a great effect on quality of the final product. The first steep water was supplemented with mixtures of antibiotics in order to selectively suppress the bacterial or fungal community. The treatments applied were adapted from previous investigations, which also showed that the effects of antibiotics were a result of their direct action on microbial metabolism without disturbing grain activity[11,18,27,28,56,57]. The present study revealed surprisingly high viable counts even after broad-spectrum antimicrobial treatments, indicating that the major part of the microbial community inside the kernel was well protected. Whereas some of the surface-attached microbes can be simply removed by changing the steep water[7], the majority of the microbial community is located between the husk and testa[46,49]. Plant-associated microbial communities typically form complex biofilms in the seed-coat tissues[37]. This multicellular mode of growth predominates in nature and provides adaptive strategies for microbes in fluctuating or stressful environments. Biofilm-grown cells have shown increased resistance to antimicrobial agents such as antibiotics[9], which could also explain the limited effects of antibiotics applied in the present study. We also showed that inhibition of one population within the complex ecosystem led to stimulated growth of non-suppressed populations. Similar results have been reported by Bol *et al.*[5].

Table IV. Effects of H_2O_2 and antimicrobial treatments on malt quality.

	Control[a]	H_2O_2[b]	Antibac[b]	Antimix[b]	Antifung[b]	Bacmix[b]
Δ Friability, %	91	+0.6	+0.9 (± 0.7)	–0.6 (± 3.2)	+0.5	–0.3
Δ Filtration time, min/250 mL)	38	–1	–11 (± 2)	–11 (± 15)	10	–13
Δ Extract, % d.w.	82.8	–0.1	+0.5 (± 0.2)	0.0 (± 0.2)	–0.1	+0.8
Δ Colour, EBC	2.7	+0.3	+0.6 (± 0.3)	0.0 (± 0.2)	–0.3	–0.6
Δ Soluble N, mg/L	912	+13	+53 (± 6)	–53 (± 5)	–70	+89
Δ Free amino N, mg/L	190	+6	+16 (± 1)	–20 (± 3)	–23	+19
Δ Wort β-glucan, mg/L	129	–30	–34 (± 16)	–19 (± 32)	+13	–23

[a] The results are an average of three malting experiments.
[b] The results for the treatments are expressed as difference (Δ) with respect to the control. The standard deviation of the differences (Δ) within the triplicate malting experiment is presented in brackets.

Table V. Effects of H_2O_2 and antimicrobial treatments on malt enzyme activities.

Enzyme activity	Control[a]	H_2O_2[b]	Antibac[b]	Antimix[b]	Antifung[b]	Bacmix[b]
Δ α-amylase, U/g	360	–14	+5 (± 16)	–26 (± 32)	–16	+14
Δ β-glucanase (30°C), endogenous, U/kg	566	–56	–8 (± 40)	–25 (± 70)	–2	+6
Δ β-glucanase (60°C), microbial, U/kg	115	–4	+8 (± 10)	+13 (± 12)	–26	+39
Δ xylanase, abs × 1000	187	–2	+7 (± 9)	–16 (± 12)	–38	+57

[a] The results are an average of three malting experiments.
[b] The results for the antimicrobial treatments are expressed as difference (Δ) with respect to the control. The standard deviation of the differences (Δ) within the triplicate malting experiment is presented in brackets.

In this study not only quantitative but also qualitative changes within the microbial community were taken into account. PCR-DGGE was demonstrated to be a useful tool to monitor population dynamics in the malting ecosystem. DNA-profiling clearly showed the differences in the bacterial community after antimicrobial treatments. This approach enabled detection of individual species as well as overall profiling of community structure with time. To our knowledge, this was the first study in which PCR-DGGE technique was applied to study microbial changes in the malting process. The results of PCR-DGGE were consistent with those obtained by culturing, and confirmed that Gram-negative bacteria were the predominant bacteria in the indigenous microbial community of native and malted barley. We identified eight different Gram-negative species belonging to the species *Erwinia, Enterobacter, Pseudomonas,* and *Rahnella.* Gram-negative bacteria are common components of plant ecosystems and appear to dominate the epiphytic bacterial communities[37]. In line with the present study, previous investigations have shown that species of *Klebsiella, Enterobacter, Serratia, Rahnella, Chromobacter, Citrobacter, Pseudomonas,* and *Xanthomonas* are common members of the malting ecosystem[20,45,46,53]. PCR-DGGE profiling revealed that unidentified bacterial species were found in the malting ecosystem. Three different, uncultured Gram-positive bacteria as well as *Agrobacterium* species were part of the predominant bacterial community of Scarlett-barley. The role of these bacteria in malting remains to be resolved.

The present study was carried out on a laboratory scale and the DGGE-profiles remained rather stable in the untreated samples during processing. The dynamics of the microbial community in the malting process is influenced by the initial barley community, interactions between the microbial populations during processing, the process conditions such as temperature and aeration, the malting equipment and selective operations such as the use of starter cultures or other additives[17,41]. Therefore, more heterogeneity would be expected on an industrial scale. For example, a substantial number of lactic acid bacteria (LAB) would be expected in commercial scale malting operations[17,41,46].

This study also showed that the large amount of barley DNA in the sample gave a strong signal in the PCR-DGGE analysis. Lopez *et al.*[34] and also Normander and Prosser[42] reported that universal bacterial primers can amplify plant chloroplast rDNA present in samples and therefore repress the PCR amplification of less dominant bacterial populations. It is obvious that amplification of non-target organisms can limit the detection of true bacterial or fungal species because the DNA from non-target organisms competes with the bacterial DNA for primers and deoxynucleoside triphosphates during PCR amplifications. It has been estimated that bacterial templates representing less than 0.1–1% of the total microbial content are not displayed in the DGGE-profiles[38]. To overcome this problem, primers can be targeted to specific microbial groups, and therefore it is possible to monitor the presence, succession and persistence of certain microbial populations within a complex community. Our further studies have also been directed to using group-specific bacterial and fungal primers to profile the microbial diversity in the malting ecosystem.

This study clearly showed that there is interaction between the microbial community and grain metabolism even with good quality, mature malting barley. By controlling microbial activity, especially bacteria, germination was improved. Even a two log reduction of aerobic bacterial counts, mainly consisting of Gram-negative species, was advantageous with respect to both grain germination and malt quality. In agreement with our results, van Campenhout and colleagues[56–58] reported that reduction of grain microbial activity led to improved controllability of germination. It has been proposed that microbes inhibit grain germination by competing with the embryo for oxygen[11,24,25,36]. Dormant grains have been especially vulnerable to microbial competition[3,11,27]. The present study

showed that in the case of normal malting barley, improved germination was not related to oxygen availability. Although both antibacterial treatments and H_2O_2 improved germination, they gave rise to different physiological responses in barley. H_2O_2, which is an active oxygen source that can diffuse directly into plant tissue[44], improved germination and led to a faster disappearance of ethanol from the malting drum headspace and to a faster gibberellic acid-dependent α-amylase synthesis. The response of aleurone cells to gibberellic acid is known to be oxygen dependent[23]. Previously we showed that oxygen deficiency, caused by applying N_2 gas during steeping, slowed down germination and led to a slower disappearance of ethanol and slower α-amylase synthesis[61]. The antibacterial treatments did not influence these parameters, although the treatments improved root emergence. Our results are supported by studies of van Campenhout and coworkers[56–58], who reported that the existence of microbial respiratory activity does not necessarily mean that there is actually competition for oxygen between plant tissues and microbes.

We suggest that the complex microbial community inside the barley layers forms a physical barrier and that reducing the bacterial load improved root emergence. This theory is supported by the results of van Campenhout *et al.*[56], who reinfected barley after antimicrobial treatment with *P. agglomerans* and detected reverse effects such as inhibited water uptake and shorter acrospires. Pseudomonads and members of Enterobacteriaceae, also identified as the predominant species in this study, produce extracellular polysaccharides and other exopolymeric substances, and are often involved in the formation of complex microbial biofilms in plant ecosystems[37]. As early as 1944, Bishop[3] suggested that bacteria developing at the time of harvest form a slime or mucus covering the embryo, and thereby inhibit grain germination. Furthermore, the gel-like polymeric matrix in plant-associated biofilms may prevent normal gas exchange and reduce diffusion of nutrients and other substances[37]. Van Campenhout *et al.*[56–58] reported that heavy microbial loads on barley grain may inhibit the plant contribution to overall CO_2 release.

This study also showed that microenvironments inside the kernel may differ from the ambient and may lead to an increase of unexpected microbial groups. In the present study, H_2O_2 treatment led to enhanced multiplication of microaerophilic lactic acid bacteria, although normally H_2O_2 is considered as an antimicrobial agent[8]. Improved seed vigour due to H_2O_2 treatment was observed as enhanced CO_2 production by the barley, and increased CO_2 concentration inside the husk layers most probably promoted LAB growth. Previous studies have also reported that an increase in aeration or in oxygen supply in steeping resulted in higher multiplication rates of LAB[33,57]. In agreement with our results, van Campenhout *et al.*[57] reported that rather than enhanced oxygen supply, the concomitant increase of CO_2 production by germinating barley explained the higher counts for LAB. This study highlights the importance of monitoring changes in the microbial community when changes are made in the process.

We also confirmed that modification of the microbial community had significant effects on the malt properties. Reduction of the bacterial community resulted in notably better lautering performance and improved extract yield. In accordance with the present study, Bol *et al.*[5] applied antifungal and antibacterial antibiotics in the malting process and showed that the reduction of bacterial activity, especially of gram-negative bacteria, reduced extract differences of malt and wort viscosity. Anderson[1] showed that the variability of filtration performance between malt batches of the same crop could be partly explained by the barley microbes. He suggested that indigenous microbes may secrete flocculents onto the surface of the malts, thus affecting the porosity of the filter beds.

The accelerated wort filtration recorded in this study could be due to the reduction of slime-forming bacteria. Our previous studies revealed that the severe mash filtration difficulties observed in the presence of split kernels were caused by heavy growth of bacteria during the malting process[22,30]. Kreisz *et al.*[29] reported that even small amounts of bacterial polysaccharides, such as gellan, levan and xanthan, had a negative impact on wort filtration. Furthermore, it has been shown that small, dead malt-derived bacteria can influence wort and beer separation and cause visible hazes in the final product[60]. The release of bacteria from the grain matrix during mashing was greatly dependent on the agitation. Therefore, differences in malt behaviour in different breweries are expected due the various types of industrial practices.

Furthermore, the good filtration rates observed after antibacterial treatments could also be due to the enhanced fungal growth and concomitant production of cell wall-degrading enzymes. Suppression of the bacterial community promoted yeast growth. A similar trend was observed in our previous study with lactic acid starter cultures[31]. Addition of starter cultures into the steeping water decreased the growth of gram-negative bacteria but increased yeast growth. We also noticed significant improvements in malt processability after starter treatments. The fungal community, especially filamentous fungi, is reported to be a significant source of cell wall-degrading enzymes such as β-glucanase and xylanase[2,6,26,41,47,58,62]. We also showed that reduction of fungal activity decreased the production of enzymes and retarded mash filtration. Van Campenhout[58] estimated that approximately 75% of malt xylanase activity was derived from the microbial community and only 25% from the grain. Our recent study[32] showed that the indigenous yeast community may also have a significant impact on the production of cell wall-hydrolysing enzymes. Fungal activity was also involved in the increased proteolysis. Several studies have reported that increased nitrogen content of wort and beer as well as enhanced wort colour have been caused by fungi, especially fusaria[2,17,21,47,50,51].

Although antimicrobial treatments based on the use of antibiotics obviously have no practical value to the malting industry as such, this study clearly indicated that malt properties could be tailored by controlling the microbial activity during malting. The indigenous microbial community of barley greatly influences grain germination and malt quality. Therefore, microflora management should be conducted in such a way that neutral or beneficial microbes are encouraged by the simultaneous suppression of harmful microbes.

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PAPER III

Yeasts in an industrial malting ecosystem

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ORIGINAL PAPER

Yeasts in an industrial malting ecosystem

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Abstract The malting ecosystem consists of two components: the germinating cereal grains and the complex microbial community. Yeasts and yeast-like fungi are an important part of this ecosystem, but the composition and the effects of this microbial group have been largely unknown. In this study we surveyed the development of yeasts and yeast-like fungi in four industrial scale malting processes. A total of 136 malting process samples were collected and examined for the presence of yeasts growing at 15, 25 and 37°C. More than 700 colonies were isolated and characterized. The isolates were discriminated by PCR-fingerprinting with microsatellite primer (M13). Yeasts representing different fingerprint types were identified by sequence analysis of the D1/D2 domain of the 26S rRNA gene. Furthermore, identified yeasts were screened for the production of α-amylase, β-glucanase, cellulase and xylanase. A numerous and diverse yeast community consisting of both ascomycetous (25) and basidiomycetous (18) species was detected in the various stages of the malting process. The most frequently isolated ascomycetous yeasts belonged to the genera *Candida*, *Clavispora*, *Galactomyces*, *Hanseniaspora*, *Issatchenkia*, *Pichia*, *Saccharomyces* and *Williopsis* and the basidiomycetous yeasts to *Bulleromyces*, *Filobasidium*, *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and *Trichosporon*. In addition, two ascomycetous yeast-like fungi (black yeasts) belonging to the genera *Aureobasidium* and *Exophiala* were commonly detected. Yeasts and yeast-like fungi produced extracellular hydrolytic enzymes with a potentially positive contribution to the malt enzyme spectrum. Knowledge of the microbial diversity provides a basis for microflora management and understanding of the role of microbes in the cereal germination process.

Keywords Barley · Malting · Yeast · Diversity · Enzyme

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Introduction

Malting, the controlled germination of cereal grains, is a complex biological process involving a wide range of biochemical and physiological reactions. The main goal is the production of various enzymes capable of degrading the grain macromolecules into soluble compounds. Malting traditionally involves three stages: steeping, germination and kilning [2]. During the steeping stage, the moisture content of the grains is increased at 14–18°C up to 43–46% by alternating immersion and air rest periods. The grains are then allowed to germinate under humid and aerobic conditions at 16–20°C for 4–6 days. Finally, germination is terminated by kilning (drying) the grains for 24 h at temperatures increasing gradually from about 50 to 85°C or more depending on the type of malt. Kilning halts biochemical reactions and ensures microbiological stability of the dried product (moisture content

3–4%). Furthermore, several colour and flavour compounds are produced during kilning. Malt, traditionally made from barley (*Hordeum vulgare*), is a key material in beer production. In addition, malt is used for the production of distilled spirits, and can also be processed into ingredients for different branches of the food industry [2].

A diverse microbial community is associated with the barley grain, and consists of various types of bacteria, yeasts and filamentous fungi [25, 49, 52]. Therefore, malting can be considered as a complex ecosystem involving two metabolically active groups: the germinating grains and the diverse microbial community. Many intrinsic and extrinsic factors including plant variety, climate, soil type, agricultural practices, storage and transport influence the richness and structure of the microbial community present in the incoming barley [15, 25, 31, 52]. Furthermore, malting conditions are extremely favourable for microbial growth [25, 49]. Steeping can be considered as a critical step in malting with respect to microbiological safety. Although some of the microbes are washed away along with steep water draining, the viable number increases tremendously during the steeping period and remains high throughout the germination period [15, 24, 25, 52]. Finally, kilning reduces microbial activity. However, the number of microbes is generally higher in malt than in native barley [49]. The microbial community is also significantly influenced by malthouse operations, and each process step can be a source of additional microbes and their metabolites [24, 51, 52]. Thus, it is evident that the interactions between grains and microbes during malting greatly influence both malting performance and the properties of the final product [25, 49]. Depending on the nature and amount of microbes, these effects may be either beneficial or detrimental [6, 14, 18, 22, 25, 28, 32, 35, 36, 46, 49, 57, 61, 68, 72].

Although several studies of microbial communities in malting have been published, most of them have focused on bacteria and filamentous fungi. Relatively little has been reported on yeasts in industrial malting ecosystems. Flannigan [25] reported that yeasts are the second most abundant microbes after bacteria in viable counts in pre-harvest barley. Furthermore, encapsulated yeasts were reported to survive during long-term storage, whereas the number of bacteria and filamentous fungi associated with barley decreased [11]. High numbers of yeasts and yeast-like fungi have also been observed during the malting process [7, 15, 24, 25, 31, 51, 52]. Traditionally the yeasts in the malting ecosystem have been approximately divided into pink yeasts and a variety of white yeasts on the basis of colony morphology [15, 24, 25]. Previously, 10 ascomycetous and 6 basidiomycetous yeast species were reported from barley and from malting samples [15, 21, 23–25, 37, 49, 52, 68, 69]. Furthermore, a yeast-like fungus *Aureobasidium pullulans* was commonly encountered in pre- and post-harvest barley samples [11, 21, 24].

The role and the effects of yeasts in the malting ecosystem are not yet fully understood. Pigmented-yeasts may be responsible for discoloration of barley kernels and of grain products [37]. Yeasts also caused bridging of damp kernels during post-harvest storage in silos [48]. This extensive formation of yeast biomass around the kernels is also very likely to affect grain physiology during malting if uncontrolled yeast growth occurs during processing. Viable yeast cells of malt origin are destroyed at the latest by the high temperatures during mashing and wort boiling in the breweries [51], but it is well known that the microbial metabolites produced during malting may survive throughout the processing and enter the final product. Kreisz et al. [38] reported that small amounts of extracellular polysaccharides produced by malt-derived bacteria and yeasts may have a negative impact on wort and beer filtration. In addition, fungal activity on the malt husk appears to create factors that influence yeast flocculation, which is an important property of brewing yeast [70]. Furthermore, yeast metabolic activity in the production chain of fermented beverages may also lead to film formation, cloudiness and haziness, sediments and excessive gas production, off-odours and -flavours at all stages of the process [10, 26, 45].

Despite their several undesired characteristics, yeasts are extremely important microbes for the food and beverage industry. By production of valuable metabolites such as enzymes and vitamins, yeasts can contribute to the processability and nutritional value of cereal products [13, 63]. However, surprisingly little is known about their possible positive contribution to malt properties. Some yeasts normally associated with malting have shown strong antagonistic activity and have been applied as natural biocontrol agents to restrict the growth of harmful fungi [6, 17]. Our previous studies with lactic acid starter cultures revealed that the addition of lactic acid bacteria (LAB) into the steeping activated the indigenous yeast community and enhanced the production of microbial β-glucanase and xylanase in the malting process [32–30, 43]. However, the source of these microbial enzymes was unknown, and we suggested that enhanced growth of the yeast community could partly explain the increased enzyme activities. To our knowledge no research has been reported on the potential of yeasts from the industrial malting ecosystem to produce extracellular hydrolytic enzymes.

The aim of this study was to investigate the diversity of yeasts and yeast-like fungi in the industrial malting ecosystem. Furthermore, yeasts isolated from the malting process were screened for the production of extracellular hydrolytic enzymes. Better understanding of yeast ecology in the malting ecosystem could lead to more efficient control of the unwanted phenomena induced by yeasts as well as to the utilization of their beneficial properties in malt production.

Materials and methods

Sample collection

Four industrial lager malt production runs were monitored during the year 2002. In all cases the Finnish malting barley cultivar Saana from the 2001 crop was used. Barley was steeped twice in conical steeping vessels at about 15°C, with an 11–18-h air rest between the steeps. After steeping, the barley was transferred to germination boxes and germinated for 5–6 days at 15–20°C. The germination process was terminated by kilning. The air temperature was first raised to 55–60°C and finally to 85°C. The total kilning time was 16–19 h. A total of 136 samples were collected at nine stages; from the original barley ($n = 4$), from the steeping vessel after the air rest ($n = 28$) and after the steeping period ($n = 13$), from the germination boxes after 1 day germination ($n = 20$) and after the germination period prior to kilning ($n = 20$), from the dryer after 5 h kilning ($n = 18$), after 10 h kilning ($n = 16$), and after the whole kilning period ($n = 14$), and the final screened malt ($n = 3$). During steeping, 1–2 kg grain samples were taken through sampling pipes positioned at various locations and depths in the steeping vessels. A grain sampler was used to collect the samples from the various locations and depths in germination boxes and during kilning.

Enumeration and isolation

Samples (10 g) were mixed with 90 ml sterile saline solution, soaked at 4°C for 30 min and homogenized with a Stomacher Lab Blender 400 (Seward Medical, London, UK). Serial dilutions of homogenate were surface plated on yeast-malt extract agar, YM-agar (Difco Laboratories, Detroit, MI, USA), which was supplemented with 0.01% chlortetracycline (Sigma, St.Louis, MO, USA) and 0.01% chloramphenicol (Sigma) to prevent bacterial growth. In addition, 0.02% Triton-X 100 (BDH Laboratory Supplies, Poole, England) was used to prevent the spreading of fungal colonies. Replicate plates were cultivated in aerobic conditions at 15, 25 and 37°C for 5 days. Counts were expressed as colony forming units per gram (cfu/g).

A total of 733 colonies were isolated from the culture plates. Selection was based on different colony morphology (diameter, shape, colour and surface). Different colony types were collected throughout the malting process in order to identify the predominant species in the malting ecosystem. Cultures were purified by cross-streaking twice on YM agar, and stored in 10% glycerol at -70° and on YM-slants at 4°C for short-term storage.

DNA extraction

Genomic DNA for PCR reactions was extracted with two different methods. In the glass bead protocol the cells were grown on YM-agar at 25°C for 2 days. A loopful (10 μl) of cell mass was suspended in 1 ml redistilled water with 0.1 g 150–212 μm glass beads (Sigma-Aldrich, St Louis, MO, USA). Samples were homogenized in a FastPrep cell disrupter (FP120, Q-Biogene, Carlsbad, CA, USA) for 2 min at 5.5 m/s. Cell debris was removed by centrifugation at 13,000 rpm for 3 min. The supernatant containing the DNA was stored at −20°C.

The simple and rapid mechanical extraction protocol with glass beads was not efficient for most of the malting yeasts. Some yeast cells from the malting process were difficult to disrupt due to their very complex cell walls and capsules. Therefore, DNA was extracted from young cultures (18–24 h) with a DNA-kit, which combined both enzymatic treatment and mechanical lysis with ceramic sphere and garnet matrix. Genomic DNA from encapsulated yeasts was extracted with FastDNA®kit (Q-Biogene, Carlsbad, CA, USA) with CLS-Y lysing solution according to the manufacturer's instructions with minor modifications. A loopful (10 μl) of cell mass was suspended with 1 ml cell lysis solution (CLS-Y) in a tube containing lysing matrix (ceramic sphere and garnet matrix). Samples were homogenized in the FastPrep cell disrupter for 3 min at 5 m/s. Cell debris was removed by centrifugation at 12,000 rpm (14,000*g*) for 10 min. Purification and elution steps were carried out according to the manufacturer's instructions. DNA samples were stored at −20°C.

Molecular typing and identification

PCR-fingerprinting with M13 microsatellite primer (5′-GAGGGTGGCGGTTCT-3′) was performed according to Andrighetto et al. [1] with minor modifications.

Five microlitres of undiluted or 1:100 diluted DNA was mixed with 45 µl of PCR master mix. The master mix contained 1× DyNAzyme reaction buffer (final concentrations 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, pH 8.8), 200 µM of each dNTP, 2 U of DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland) and 1 µM of M13 primer (Sigma-Genosys, Cambridge, UK). Amplification was carried out in a UNO II Thermal Cycler (Biometra GmbH, Goettingen, Germany), with initial denaturation at 95°C for 5 min followed by 34 cycles of 60 s at 94°C, 20 s at 45°C (ramping to 72°C at 0.5°C–s^{-1}), 60 s at 72°C and 20 s at 50°C and a final extension at 72°C for 10 min. A reagent control in which DNA was replaced with redistilled water was included in every experiment. Amplified DNA fragments were separated by electrophoresis in 1.8% agarose gel (Cambrex Bio Science Rockland Inc., Rockland, ME, USA) in 0.5× TBE (Tris–Borate–EDTA, Bio-Rad laboratories, Hercules, CA, USA) buffer at 120 V for 4 h, and visualized by fluorescent labelling with ethidium bromide (Mercury, CLP, San Diego, CA, USA). Similarities between the DNA fingerprints of the selected isolates were determined with the aid of the Bionumerics program using the Unweighted Pairgroup Method with Arithmetic averages (UPGMA) clustering based on the Pearson correlation.

The strains were identified by sequence analysis of the D1/D2 domain of the 26S rRNA gene as described by Kurtzman and Robnett [40] except that the amplified DNA was purified with a QIAquick PCR purification kit (Qiagen, Mississaugua, Ontario, Canada), and the ABI BigDye v3.1 Terminator Cycle Sequencing kit was used for sequencing reactions (Applied Biosystems, Foster City, CA, USA). Electrophoresis of the products was carried out in an ABI PRISM 3100 Genetic Analyser (Applied Biosystems). The sequences were edited with DNAMAN software version 4.1 (Lynnon Biosoft, Que, Canada). For identification, the sequences were compared with those of all known species available at the GenBank Database. A similarity of > 99% to 26S rRNA gene sequences was used as a criterion for identification [40]. Identified strains were deposited in the VTT Culture Collection and their nucleotide sequences were deposited in GenBank under the accession numbers shown in Tables 2 and 3.

Production of extracellular hydrolytic enzymes

The production of extracellular hydrolytic enzymes was studied with a plate-screening method. Yeast strains (27 ascomycetous and 28 basidiomycetous yeast) were cultivated on YNBG plates containing 0.67% yeast nitrogen base (YNB, Difco Laboratories), 1% glucose and 2% agar at 25°C for 2 days. Yeasts were then surface streaked on duplicate enzyme assay plates and incubated at 25°C for 3 days. Strains were screened for their ability to hydrolyse starch on a medium (YNBS) containing 0.67% YNB, 2% agar and 1% soluble starch (Merck, Darmstadt, Germany). After cultivation, the plates were stained with Lugol solution (containing iodine, 1 g; potassium iodine, 2 g and distilled water 300 ml). A yellow zone around a colony, in blue-stained medium, indicated amylase activity. β-Glucanase activity was determined on YNB plates (YNBB) supplemented with 0.5% barley β-glucan (P-BGBM, Megazyme, Wicklow, Ireland). Plates were flooded with 0.1% Congo red solution for 10–15 min and excess dye was rinsed off with 1 M NaCl. Clear halos around the colonies indicated hydrolysis of the substrate. Cellulase production was determined on YNBC plates containing 0.5% carboxymethylcellulose (CMC, Fluka, Buchs, Switzerland). For screening of hemicellulase activity, the YNB plates (YNBX) were supplemented with 1% xylan (Xylan from oat spelts, Sigma). The presence of extracellular cellulases and xylanase was detected with the Congo red method as described above.

Results and discussion

Yeast growth in the industrial malting ecosystem

The yeast community was monitored in four batches of Saana–barley from the 2001 crop as they went through the industrial malting processes from steeping to kilning (Table 1). The initial yeast count on stored barley was 2×10^4–2×10^5 cfu/g. The soaking of barley activated yeast growth, and a 10–100-fold increase of yeast counts was detected during the first days of malting. O'Sullivan et al. [51] reported an increase of the same magnitude in industrial scale, and Petters et al. [52] observed a 1,000-fold increase of yeasts during an industrial steeping period. Yeast activity remained high throughout the germination period, and the viable count reached a maximum level of 10^7 cfu/g at the end of germination.

We also studied the effect of growth temperature on the yeast counts. All the samples were incubated at 15, 25 and 37°C (Table 1). The majority of yeasts live in habitats in which the temperature domain is between 0 and 45°C [12]. As shown in Table 1, yeasts in the malting ecosystem were capable of growing at 15°C. Our results were in agreement with those of Petters et al.

Table 1 Yeast growth (cfu/g) during the industrial scale malting processes

Malting phase	*N*	Incubation temperature					
		15°C		25°C		37°C	
		Mean	Range	Mean	Range	Mean	Range
Barley	4	2×10^5	1×10^5–2×10^5	7×10^4	2×10^4–2×10^5	<50	<50
Steep, after air rest	28	4×10^5	1×10^5–7×10^5	3×10^5	3×10^4–1×10^6	1×10^3	<50–6×10^3
After steeping period	13	2×10^5	2×10^5–3×10^5	7×10^5	8×10^4–2×10^6	8×10^2	2×10^2–2×10^3
After 1 day germination	20	2×10^6	5×10^5–5×10^6	3×10^6	3×10^5–9×10^6	5×10^3	4×10^2–3×10^4
After germination	20	3×10^7	1×10^7–7×10^7	3×10^7	1×10^7–5×10^7	5×10^5	7×10^3–5×10^6
After 5 h kilning	18	4×10^7	2×10^7–6×10^7	3×10^7	1×10^7–6×10^7	6×10^5	4×10^3–4×10^6
After 10 h kilning	16	3×10^7	4×10^6–7×10^7	2×10^7	4×10^6–7×10^7	5×10^5	2×10^3–2×10^6
After kilning	14	2×10^6	9×10^5–3×10^6	2×10^6	7×10^5–4×10^6	3×10^4	<50–9×10^4
Screened malt	3	1×10^6	4×10^5–2×10^6	1×10^6	5×10^5–1×10^6	9×10^4	4×10^2–2×10^5

[52], who also found that the occurrence of yeasts in malting was attributed to their ability to grow at low temperatures.

Malting processes also harboured yeasts capable of growing at 37°C (Table 1). After the steeping periods 2×10^2–2×10^3 cfu/g were detected, and the viable count increased significantly during the germination and the initial phase of kilning. Thermotolerant yeasts represented ~2% of the yeast community after germination. Greater variation in the number of thermotolerant yeasts was observed within the batches compared to the populations growing at 15 or 25°C. Thermotolerant yeasts obviously originated from the malting equipment, as they were not detected in the native barley samples. Batch-to-batch variation in the process environment and in the malting procedures could explain the observed fluctuation. It has been shown that a specific microbial community develops in each malting plant [51, 52]. Furthermore, the yeast community present in the malting process depends on the initial population of barley, interactions between the different microbial groups, process conditions such as temperature and aeration, and the use of antimicrobial treatments such as starter cultures [25, 43, 49, 52].

Kilning appeared to have little effect on viable counts (Table 1). Kilning started with an air-on temperature of 55–60°C. Barley dried progressively from the bottom to the top of the grain bed, and the time that barley was exposed to each temperature depended on its location in the kiln. The first hours of kilning before the temperature breakthrough, especially in the top layers of the grain bed, appeared to favour yeast growth (data not shown). Only a tenfold reduction in yeast counts was observed during kilning. Yeast numbers in the finished malt ranged from 4×10^5 to 1×10^6 cfu/g. Schwarz et al. [60] also reported a large increase in ergosterol content during the early hours of kilning, indicating that the fungal growth was accelerated. Therefore, kilning can also be regarded as an important step with regard to the microbiological quality of malt. The kilning regime has been identified as a significant factor in controlling the microbial community [62]. Under normal environmental conditions, the vegetative yeast cells are rapidly inactivated by temperatures of 60–65°C [26]. However, in the malting ecosystem yeasts are well protected under the outer layers of the barley. Microbial populations adhere to external and internal surfaces of grain tissues to form a compact biofilm, which protects cells against heat and other antimicrobial treatments [66]. As discussed later, a large proportion of the yeast community was composed of encapsulating yeasts, which could also explain the high number of survivors in the kilned malt.

Characterization and identification of yeasts in the industrial malting ecosystem

The main goal of this study was to obtain an overall picture of the yeasts present in the industrial malting ecosystem. We isolated over 700 yeast colonies from various stages of the process. All the isolated yeasts were detected at least at a level of 10^4–10^5 cfu/g. Furthermore, the selection of different colony types from several samples throughout the malting process ensured that the predominant species were selected. However, some minor species may have been overlooked on the plates.

We applied DNA-based techniques for the differentiation of yeast isolates and for species identification. Yeast isolates were first discriminated with PCR-fingerprinting using an oligonucleotide primer (M13), targeting simple repetitive DNA sequences named microsatellites. This protocol has been widely applied in yeast typing and it allows discrimination of species even at the subspecies level [44]. In this study 55 fingerprint patterns were detected. All the yeast isolates with different DNA-fingerprints were further identified with sequencing of

the DNA sequence (600–650 nucleotides) from the 5′ end of the 26S rRNA gene, D1/D2 region [19, 40].

A surprisingly diverse yeast community was detected in the industrial malting processes of Saana-01 barley. We detected 25 species of ascomycetous yeasts belonging to eight different genera (Table 2) and 18 species of basidiomycetous yeasts belonging to six different genera (Table 3). The most frequently encountered ascomycetous genera were *Candida*, *Clavispora, Galactomyces, Hanseniaspora, Issatchenkia, Pichia, Saccharomyces* and *Williopsis* (Table 2). In addition, two ascomycetous yeast-like fungi or black yeasts, *A. pullulans* and *Exophiala dermatidis*, were frequently detected. *Candida* species were allocated to 11 different species, namely *C. anglica*, *C. cylindracea*, *C. fermentati*, *C. intermedia*, *C. natalensis*, *C. pararugosa*, *C. picinguabensis*, *C. saitoana*, *C. sake*, *C. silvae* and *C. solani*. Furthermore, two sets of isolates *Candida* sp. I (C-04530) and II (C-04532) did not match closely enough to any of the sequences present at the time in the database query. *Candida* sp. I gave only 92.8% similarity to an undescribed *Candida* sp. and strain IV 93.9% to *Issatchenkia* sp. These two isolates may represent novel *Candida* or *Issatchenkia* species and are being subjected to further characterization.

Basidiomycetous yeasts comprised six different genera: *Bulleromyces, Cryptococcus, Filobasidium, Rhodotorula, Sporobolomyces* and *Trichosporon* (Table 3). A wide variety of white, cream and pigmented *Cryptococcus* species were detected in the malting ecosystem. They were identified as *C. albidosimilis, C. curvatus, C. hungaricus, C. macerans, C. magnus, C. victoriae* and *C. wieringae*. In addition, four groups of undescribed *Cryptococcus* species, indicated as *Cryptococcus* sp. I–IV, were found on the basis of D1/D2 sequences. *Cryptococcus* sp I (VTT C-04545) showed 99.4%

Table 2 Ascomycetous yeasts identified by sequence analysis of the 26S rRNA D1/D2 region

Genera	Species	VTT Culture collection number	Sequence similarity to the closest species/strain		GeneBank Account number
			%	bp	
Candida	*C. anglica*[a], *smooth colony type*	C-04516	100	570/570	DQ377631
	C. anglica[a], *rough colony type*	C-04517	100	570/570	DQ377632
	C. cylindracea	C-04529	99.1	568/573	DQ377633
	C. fermentati	C-04519	100	534/534	DQ377634
	C. intermedia	C-04520	99.8	518/519	DQ377635
	C. natalensis	C-04521	100	569/569	DQ377636
	C. pararugosa	C-04522	100	581/581	DQ377637
	C. picinguabensis	C-04523	100	488/488	DQ377638
	C. saitoana	C-04524	100	538/539	DQ377639
	C. sake	C-04518	100	587/587	DQ377640
	C. silvae	C-04527	100	541/541	DQ377641
	C. solani	C-04528	100	568/568	DQ377642
	Candida sp. II	C-04530	92.8	323/348	DQ377643
	Candida sp. IV	C-04532	93.9	526/560	DQ377644
Clavispora	*Cl. lusitaniae*	C-04533	99.8	551/552	DQ377645
Galactomyces	*G. geotrichum*[b]		nd	nd	
	Geotrichum silvicola	C-04559	99.6	559/561	DQ377646
Hanseniaspora	*H. clermontiae/meyri*	C-04560	99.8[c]	583/584	DQ377647
	H. uvarum	C-04561	100	556/556	DQ377648
Issatchenkia	*I. orientalis*	C-04562	100	602/602	DQ377649
Pichia	*P. anomala*	C-04565	100	573/573	DQ377650
	P. fabianii	C-04566	100	613/613	DQ377651
	P. fermentans	C-04567	100	556/556	DQ377652
	P. guilliermondii	C-04568	100	561/561	DQ377653
Saccharomyces	*S. exiguous*	C-04572	99.5	586/589	DQ377654
Williopsis	*W. californica*	C-04576	100	632/632	DQ377655
Black yeasts					
Aureobasidium	*A. pullulans*	D-041013	100	614/614	DQ377656
Exophiala	*E. dermatidis*	D-041016	100	617/617	DQ377657

[a] Two different colony types of *C. anglica* were deposited in the VTT culture collection although they gave similar DNA-fingerprinting patterns and identical sequences

[b] *G. geotrichum* was identified on the basis of morphology and DNA-fingerprints. Pure cultures, previously isolated and identified from the industrial malting processes (VTT C-94425 and VTT C-99718), were compared to those isolated in this study

[c] Could not be separated by D1/D2 sequencing

Table 3 Basidiomycetous yeasts identified by sequence analysis of the 26S rRNA D1/D2 region, VTT Culture Collection number and GeneBank access number

Genera	Species	VTT Culture collection number	Sequence similarity to the closest species/strain		GeneBank Account number
			%	bp	
Bulleromyces	*B. albus*	C-04514	100	632/632	DQ377658
Cryptococcus	*C. albidosimilis*	C-04508	99.8	611/612	DQ377659
	C. curvatus	C-04536	99.9	507/508	DQ377660
	C. hungaricus	C-04558	98.8	619/629	DQ377661
	C. macerans	C-04538	99.8	624/625	DQ377662
	C. magnus	C-04540	99.8	643/644	DQ377663
	C. victoriae	C-04542	100	489/489	DQ377664
	C. wieringae	C-04509	100	626/626	DQ377665
	Cryptococcus sp.I	C-04545	99.4	618/622	DQ377666
	Cryptococcus sp.II[a]	C-04546	99.8	602/603	DQ377667
		C-04547	100	603/603	DQ377668
	Cryptococcus sp.III[a]	C-04548	99.8	628/629	DQ377669
		C-04549	99.8	639/640	DQ377670
		C-04550	99.8	634/635	DQ377671
		C-04551	99.4	637/641	DQ377672
	Cryptococcus sp.IV[a]	C-04510	100	532/532	DQ377673
		C-04552	100	611/611	DQ377674
		C-04553	100	611/611	DQ377675
		C-04554	100	611/611	DQ377676
		C-04555	100	611/611	DQ377677
		C-04556	100	611/611	DQ377678
		C-04557	100	611/611	DQ377679
Filobasidium	*F. globisporum*	C-04511	99.4	627/631	DQ377680
Rhodotorula	*R. glutinis*	C-04513	100	617/617	DQ377681
	R. pinicola	C-04570	100	626/627	DQ377682
Sporobolomyces	*S. roseus*	C-04574	100	604/604	DQ377683
	S. ruberrimus	C-04573	100	597/597	DQ377684
Trichosporon	*T. brassicae*	C-04575	100	626/626	DQ377685

[a] Members of the *Cryptococcus* II–IV groups gave several banding patterns with microsatellite primers and strains with distinct fingerprints have been deposited to VTT culture collection

similarity to *Cryptococcus* strain CBS 7743 (Gene bank no. AJ311452), which was closely related to *C. nyarrowii*. These strains were previously isolated from soil and snow from Antarctica [67]. The D1/D2 sequences of the *Cryptococcus* sp. II isolates were identical with those of *Cryptococcus* yeasts isolated from soil in Austria (HB946, Genbank no. AJ510201), and for the isolates of the type III the closest relative was another *Cryptococcus* HB1052 strain isolated from soil in Austria (Genbank no AJ510146). The sequence of the *Cryptococcus* sp. IV was identical with that of the strain KCTC 17065 (Genbank no. AF459681) isolated from flower samples in Korea [33]. *Cryptococcus* isolates I–IV have been subjected to further phenotypic and genotypic characterization.

Occurrence of yeasts in the various stages of the malting process

The species detected in the various stages of the malting process are summarized in Table 4. Basidiomycetous yeasts dominated in the yeast community of barley. In addition they were frequently detected during the first days of malting. The growth of basidiomycota was favoured by low temperatures during steeping. Many species have temperature optima below 20°C [12, 67]. The oxidative basidiomycetous yeasts are common in plant ecosystems [25, 27]. The attachment of these yeasts to plant surfaces is attributed to the production of extracellular gums and mucilages, which also protects the cells from desiccation and other external factors [8]. This study clearly shows that encapsulated basidiomycetous yeasts can survive the high temperatures reached during kilning. Basidiomycetous yeasts also have other survival properties, such as the ability to compete with other organisms for nutrients, which could their abundance in the barley ecosystem [27]. In addition, *Cryptococcus, Rhodotorula* and *Sporobolomyces* generally produce red, pink and yellow carotenoid pigments, which enhance tolerance to sunlight and radiation [27]. Yeast pigments may later be involved in the discoloration of grains and cereal products.

Table 4 The predominant yeast species in the various stages of malting

Barley	Steeping		Germination		Kilning		
	After air rest	After steeping	After 1 day germination	After germination	After 5 h kilning	After 10 h kilning	Malt isolates
isolates (*n* = 20)	Isolates (*n* = 113)	Isolates (*n* = 104)	Isolates (*n* = 119)	Isolates (*n* = 79)	Isolates (*n* = 101)	Isolates (*n* = 84)	(*n* = 113)
Ascomycetous yeasts							
A. pullulans	*C. anglica* *C. fermentati*[a] *C. intermedia*[a] *C. natalensis* *C. pararugosa*[a] *C. silvae* *P. fabianii*[a] *P. fermentans*[a] *A. pullulans*	*C. anglica* *C. fermentat*[a] *C. pararugosa* *Candida sp. I*[a] *G. geotrichum* *P. anomala* *P. fermentans*[a] *P. guilliermondii*[a] *W. californica* *A. pullulans* *E. dermatidis*	*C. anglica* *C. natalensis* *C. pararugosa*[a] *C. solani* *Cl. lusitaniae*[a] *G. geotrichum* *P. guilliermondii* *W. californica* *A. pullulans* *E. dermatidis*	*C. anglica* *C. fermentati*[a] *C. natalensis* *C. pararugosa*[a] *C. saitoana* *C. silvae* *Cl. lusitaniae*[a] *G. geotrichum* *H. clermontiae* *I. orientalis*[a] *P. anomala* *P. fabianii*[a] *P. fermentans*[a] *P. guilliermondii*[a] *W. californica*	*C. anglica* *C. cylindracea* *C. natalensis* *C. pararugosa*[a] *C. picinguabensis*[a] *C. saitoana* *C. silvae* *C. solani* *Cl. lusitaniae*[a] *G. geotrichum* *H. clermontiae* *H. uvarum* *I. orientalis*[a] *P. anomala* *P. fabianii*[a] *P. fermentans*[a] *P. guilliermondii*[a] *S. exiguus* *A. pullulans* *E. dermatidis*	*C. anglica* *C. cylindracea* *C. pararugosa* *C. sake* *C. saitoana* *C. silvae* *Cl. lusitaniae*[a] *H. uvarum* *P. anomala* *P. fabianii*[a] *P. fermentans*[a] *P. guilliermondii*[a] *A. pullulans*	*C. anglica* *C.natalensis* *C. pararugosa*[a] *C. saitoana* *C. silvae* *Candida sp. II* *Cl.lusitaniae*[a] *G. geotrichum* *G. silvicola* *H. uvarum* *I. orientalis*[a] *P. fabianii*[a] *P. fermentans*[a] *P. guilliermondii*[a] *A. pullulans*
Basidiomycetous yeasts							
B. albus *C. albidosimilis* *C. magnus* *C. wieringae* *Cryptococcus* sp IV *F. globisporum* *R. glutinis* *S. ruberrimus*	*B. albus* *C. albidosimilis* *C. hungaricus* *C. macerans* *C. victoriae* *C. wieringae* *Cryptococcus sp I* *Cryptococcus* sp IV *F. globisporum* *R. glutinis* *S. ruberrimus* *T. brassicae*[a]	*B. albus* *C. hungaricus* *C. victoriae* *C. wieringae* *Cryptococcus* sp II *Cryptococcus* sp III *Cryptococcus* sp IV *F. globisporum* *R. glutinis* *S. ruberrimus*	*B. albus* *C. albidosimilis* *C. curvatus*[a] *C. victoriae* *C. wieringae* *Cryptococcus* sp II *Cryptococcus* sp III *Cryptococcus* sp IV *F. globisporum* *R. glutinis* *S. ruberrimus*	*Cryptococcus* sp III *Cryptococcus* sp IV *R. glutinis* *S. roseus* *S. ruberrimus*	*Cryptococcus* sp III *Cryptococcus* sp IV *R. glutinis* *R. pinicola* *S. ruberrimus*	*C. magnus* *Cryptococcus* sp III *Cryptococcus* sp IV *R. glutinis* *R pinicola* *S. ruberrimus*	*B. albus* *C. macerans* *C. magnus* *Cryptococcus* sp IV *R. glutinis* *S. ruberrimus*

[a] Species detected after 37°C cultivation

The following six basidiomycetous yeasts: *C. albidus, Cryptococcus* sp. *R. glutinis, R. mucilaginosa, S. roseus* and *T. beigelii* have previously been reported in the malting process [15, 21, 23, 25, 49, 52, 69]. We found 11 different *Cryptococcus* species in the malting process including four potentially novel species. *Cryptococcus* species are commonly found in both natural and man-made ecosystems. *Cryptococcus* species as well as *Filobasidium globisporum* were encountered especially during steeping and at the beginning of germination. *F. globisporum* is also known from plant material and is often found in weathered leaves [4, 39]. This study showed that *Bulleromyces albus* (anamorph *Bullera alba*), commonly associated with plant ecosystems [4], also survived throughout the malting process.

In agreement with previous investigations [24, 25, 49, 52, 69], the red-pigmented yeasts *Rhodotorula* and *Sporobolomyces* were important members of the malting ecosystem. *Rhodotorula* species can be found from a variety of substrates and environments world-wide [39]. *Sporobolomyces* species mainly occur in the phyllosphere [5, 20]. *R. glutinis* was isolated in every malting sample (Table 4). In addition we detected *R. pinicola* in samples from the initial phase of kilning. This species was recently isolated from pine twigs [74]. We detected the presence of two *Sporobolomyces* species during malting. *S. ruberrimus* was isolated at every stage and *S. roseus* at the end of germination. We also isolated one member of the *Trichosporon* genus during steeping; *T. brassicae*, which has previously been detected from cabbage [39].

In contrast to basidiomycetous yeasts, ascomycetous species dominated at the end of germination and especially during the first hours of kilning. We found 20 different ascomycetous yeasts in the samples taken after 5 h of kilning, whereas only five basidiomycetous species were detected in the same samples (Table 4). The occurrence of ascomycetous yeasts was obviously due to their ability to grow better at the higher temperatures prevailing at the end of malting than basidiomycetous yeasts. The predominant yeast species detected in 37°C cultivations were *C. fermentati, C. intermedia, C. pararugosa, Candida* sp I, *Cl. lusitaniae, I. orientalis, P. fabianii, P. fermentans* and *P. guilliermondii*. Only two basidiomycetous yeasts, *C. curvatus* and *T. brassicae*, were detected in YM-plates cultivated at 37°C.

Candida species were among the most frequently detected ascomycota throughout the malting process. This study revealed 13 different species in the malting ecosystem. Only three different species have previously been reported from malting processes [25, 49, 52]. *C. anglica, C. pararugosa, C. natalensis* and *C. silvae* were present in every malting stage including the final malt. The genus *Candida* is an extremely heterogenous group of yeasts and is commonly associated with plants, rotting vegetation, insects which feed on plants, or with different foods and beverages [39, 41, 54, 58].

This study showed that *Clavispora lusitaniae* was part of the malting ecosystem and was detected in samples derived from germination and kilning. It was frequently isolated from 37°C cultivations. *C. lusitaniae* occurs in a wide variety of substrates of plant and animal origin as well as in industrial wastes and clinical specimens [39, 42].

Galactomyces geotrichum (anamorph *Geotrichum candidum*) was a common isolate in process samples. In addition to *G. geotrichum* another *Geotrichum* species, *G. silvicola*, was isolated from the malt samples (Table 4). *G. geotrichum* is generally considered as a process contaminant and is rarely present in native barley, but may become one of the dominant fungi during germination and is often found in kilned products [52]. Douglas and Flannigan [15] reported that increased aeration during steeping may promote the growth of *Geotrichum*. They also reported that contamination with *G. geotrichum* led to reduction of the other yeast-like fungi. Subsequently, *G. geotrichum* with antifungal properties has found an application in the malting industry as a biocontrol agent for prevention of toxigenic fungi [6].

Members of the genus *Hanseniospora* became part the yeast community at the end of germination and were detected particularly during the initial phase of kilning. We detected two different species, *H. clermontiae* and *H. uvarum*, which has previously been reported in the malting process [52]. Furthermore, species of *Hanseniaspora* are common organisms in soil, various plants and fruits and are particularly associated with grapes and wine production [27, 53].

Issatchenkia orientalis was isolated from the samples derived from germination and kilning. It also entered the final product. *I. orientalis* is also commonly associated with various foods such as fruit juice, tea, beer, bread, dairy products, fermented foods and beverages [39]. It has often been linked with food and beverage spoilage and particularly with film formation [53].

The *Pichia* population during malting was composed of four species: *P. anomala*, *P. fabianii, P. fermentans* and *P. guillliermondii*. All of them were detected in every stage of the process except for *P. anomala*, which was isolated only during the first hours of kilning. *Pichia* are also an extremely heterogenous group of yeasts [39]. They are common in both natural and clinical environments and are often found in industrial fermentation processes [3, 39]. *P. guilliermondii* is often isolated from insects such as beetles, which are the

principal vectors for the transportation of yeasts in plant ecosystems [65]. *P. anomala* and *P. guilliermondii* strains with antagonistic activity have been applied as biocontrol agents to suppress pre- and post-harvest fungal diseases [16, 55].

Only one representative of the genus *Saccharomyces*, *S. exiguus* was detected during the first hours of kilning. This species is often associated with fresh and processed vegetables as well as with spoilage of soft drinks and fruit juices [39]. It is also a common organism in sour doughs [12]. *Williopsis californica* was mainly found in the samples derived from steeping and germination. This species is widely distributed in nature, commonly being found in soil, streams, lakes and plants [39].

Ascomycetous yeast-like fungi were frequently encountered in the malting ecosystem. *A. pullulans* was already present in the indigenous microbial community of barley, and was also detected in every process step (Table 4). Several studies have shown that *A. pullulans* is a very common organism in pre- and post-harvest barley samples as well as in the malting process [15, 21, 22, 24, 52]. *A. pullulans* is a ubiquitous saprophyte in the phyllosphere and is often found in decaying and damp materials [53, 73]. It has been reported from various foods, but only rarely linked to food spoilage [53]. It is also a potential biocontrol agent of plant pathogens [59].

The other black yeast, *E. dermatidis*, was detected in samples derived from steeping, germination and kilning, but not in the native barley. Thus, it can be considered as a process contaminant. *E. dermatidis* has a world-wide distribution and has been isolated especially from environments with high temperature, high moisture and low nutrient levels such as bathrooms, saunas and steam rooms [47]. The cells are protected by extracellular polysaccharides, which promote their survival in harsh environments. These properties could also explain their frequent occurrence in the malting environment. Although this organism is commonly found in various man-made environments, its natural ecological niche is unknown [47].

It is evident that a great number of different yeast species play a significant role in the industrial malting ecosystem, and that yeast association with grains may be more important than previously believed. In this study nine species were predominant in native barley and 21 different species formed the prevalent yeast community in the screened malt. Although only one cultivar from one crop year was followed throughout the processing, this study provides a clear indication of the vast microbial diversity in commercial scale malting. It is obvious that even more heterogeneity could be expected due to differences between crops as well as between industrial practices in different locations.

Production of extracellular hydrolytic enzymes

Malted grains can be considered as packages of enzymes and nutrients utilized especially in beer production. The successful production of malt includes production of various hydrolytic enzymes and controlled degradation of the grain endosperm structure. The key component of barley of interest to the brewer is the starchy endosperm, which represents about 70% of the total weight [2]. The cell walls of barley contain 70% [1, 3], (1,4)-β-D-glucan, 20% arabinoxylan, 6% protein, 2% cellulose and small amounts of other components [71]. It is now recognised that the microbial community associated with the grains has a significant impact on malt enzyme potential. Several studies have indicated that filamentous fungi present in barley and in malting produce a wide range of enzymes [22, 23, 35, 50, 56, 61, 72]. Although yeasts are important players in the malting ecosystem, their contribution to malting and brewing performance is still not fully understood. In this study, yeasts and yeast-like fungi isolated from industrial malting processes were screened for the production of amylase, β-glucanase, cellulase and xylanase. To our knowledge this is the first report on enzyme profiles of yeasts isolated from the malting environment.

The enzyme profiles were determined using minimal medium containing 0.5 -1% of substrate as sole carbon source (Tables 5 and 6). All the yeast grew in YNB supplemented with glucose, which was used as a positive control. Ascomycetous yeasts in general were not able to utilise complex polysaccharides as the only source of energy in the plate-screening assay. An exception was the yeast-like fungus *A. pullulans*, which was an effective degrader of all the substrates tested. In addition, *E. dermatidis* hydrolysed both barley β-glucan and carboxymethylcellulose. We also found that *G. silvicola* (C-04559) degraded cellulose. As shown in Table 6, basidiomycetous yeasts were active in the production of glucanase, cellulase and xylanase. Several basidiomycetous yeasts also hydrolysed starch. The most intensive degradation was observed with *C. macerans*, *S. roseus* and *S. ruberrimus* isolates. In addition, several species exhibited β-glucanase activity. The most significant producers of β-glucanase were *B. albus, C. macerans, C. magnus*, *F. globisporum* and *R. pinicola.* All these species, except *R. pinicola*, also showed cellulase activity. Cellulose and xylan degradation was detected especially among the *Cryptococcus* species. Many of these activities are assumed to be

Table 5 Production of extracellular enzymes by ascomycetous yeasts isolated from an industrial malting ecosystem

Genera	Species	VTT number	Amylase	β-Glucanase	Cellulase	Xylanase
Candida	*C. anglica*	C-04516	–	–	–	–
	C. anglica	C-04517	–	–	–	–
	C. cylindracea	C-04529	–	–	–	–
	C. fermentati	C-04519	–	–	–	–
	C. intermedia	C-04520	–	–	–	–
	C. natalensis	C-04521	±	–	–	–
	C. pararugosa	C-04522	–	–	–	–
	C. picinguabensis	C-04523	–	–	–	–
	C. saitoana	C-04524	–	–	–	–
	C. sake	C-04518	–	–	–	–
	C. silvae	C-04527	–	–	–	–
	C. solani	C-04528	–	–	–	–
	Candida sp I	C-04530	–	–	–	–
	Candida sp II	C-04532	–	–	–	–
Clavispora	*C. lusitaniae*	C-04533	–	–	–	–
Geotrichum	*G. silvicola*	C-04559	–	–	+++	–
Hanseniaspora	*H. clermontiae.*	C-04560	–	–	–	–
	H. uvarum	C-04561	–	–	–	–
Issatchenkia	*I. orientalis*	C-04562	–	–	–	–
Pichia	*P. anomala*	C-04565	–	–	–	–
	P. fabianii	C-04566	–	–	–	–
	P. fermentans	C-04567	–	–	–	–
	P. guilliermondii	C-04568	–	–	–	–
Saccharomyces	*S. exiguus*	C-04572	–	–	–	–
Williopsis	*W. californica*	*C-04576*	–	–	–	–
Black yeasts						
Aureobasidium	*A. pullulans*	D-041013	+++	+++	+++	++
Exophiala	*E. dermatidis*	D-041016	–	+++	++	–

+...++ positive result: a clear zone, +++ intensive degradation of the substrate, – negative result: no degradation, ± weak production

necessary for the fungi to degrade barley cell wall components and to penetrate plant cell walls [22].

In agreement with earlier research [9, 64], this study shows that yeasts are potential producers of enzymes degrading plant cell walls. However, some enzyme activities, especially those of ascomycetous yeasts, may have been overlooked in the plate screening method in which the complex carbohydrate is the only source for energy. Strauss et al. [64] reported that some ascomycetous yeasts showed cellulase activity only in the presence of glucose. Horn [34] reported that growth of the ascomycetous yeast *Pichia guilliermondii* associated with corn was considerably increased in association with amylolytic filamentous fungi. In the malting ecosystem complex interactions with the other microbial groups such as filamentous fungi and bacteria as well as with the germinating grain influence the growth and activity of the yeast community. Yeasts may also be a source of proteolytic and lipolytic activities. Although these enzymes have not been as extensively studied as polysaccharide hydrolysing enzymes, they are known to influence malt quality. Our further work will include studies on the microbial contribution to these enzymes.

Conclusions

This study revealed that the indigenous yeast community in the industrial malting ecosystem is complex and consists of a wide variety of ascomycetous and basidiomycetous yeasts. Although some minor species may have been overlooked in this study, the diversity of the yeast community in barley malting is greater than expected. Some potentially novel species were found in the malting ecosystem. Many of the yeasts associated with barley and malting produced enzymes degrading plant cell walls, which may contribute to the enzyme potential of malt, although their significance in industrial malt production needs to be confirmed. Knowledge of the microbial ecology of the malting process provides a basis for microflora management and understanding of the role of microbes in the malting ecosystem.

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Table 6 Production of extracellular enzymes by basidiomycetous yeasts isolated from an industrial malting ecosystem

Genera	Species	VTT number	Amylase	β-Glucanase	Cellulase	Xylanase
Bulleromyces	*B. albus*	C-04507	+/−	+++	+++	+
Cryptococcus	*C. albidosimilis*	C-04508	+/−	+/−	−	+
	C. curvatus	C-04536	−	+	−	+
	C. hungaricus	C-04558	++	−	−	−
	C. macerans	C-04538	+++	+++	+++	−
	C. magnus	C-04540	−	++	+++	+
	C. victoriae	C-04542	−	−	−	+
	C. wieringae	C-04509	+	−	+++	+
	Cryptococcus sp.I	C-04545	+	−	+++	−
	II	C-04546	+	−	+++	+/−
		C-04547	+	−	+++	+/−
	III	C-04548	−	−	+++	+
		C-04549	−	−	+++	+
		C-04550	−	+/−	+++	+
		C-04551	−	−	−	−
	IV	C-04510	−	−	−	+
		C-04552	−	−	−	+
		C-04553	−	+	−	+
		C-04554	−	−	−	+
		C-04555	−	−	−	+
		C-04556	−	−	−	+
		C-04557	−	−	−	+
Filobasidium	*F. globisporum*	C-04511	−	+++	+++	−
Rhodotorula	*R. glutinis*	C-04513	−	−	−	−
	R. pinicola	C-04570	−	++	−	−
Sporobolomyces	*S. roseus*	C-04574	+++	−	−	−
	S. ruberrimus	C-04573	+++	−	−	−
Trichosporon	*T. brassicae*	C-04575	−	−	−	−

+... + + positive result: a clear zone, + + + intensive degradation of the substrate, − negative result: no degradation, + /− weak production

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PAPER IV

Yeasts isolated from industrial maltings can suppress *Fusarium* growth and formation of gushing factors

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Yeasts isolated from industrial maltings can suppress *Fusarium* growth and formation of gushing factors

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ABSTRACT

Fusarium infection of barley and malt can cause severe problems in the malting and brewing industry. In addition to being potential mycotoxin producers, *Fusarium* fungi are known to cause beer gushing (spontaneous overfoaming of beer). Cereal-derived bacteria and yeasts are potential biocontrol agents. In this study, the antifungal potential of selected yeasts (12 strains) derived from the industrial malting ecosystem was studied *in vitro* with a plate-screening assay. Several ascomycetous yeast strains showed antagonistic activity against field

and storage moulds, *Pichia anomala* being the most effective strain. The effects of *P. anomala* VTT C-04565 (C565) were examined in laboratory scale malting with naturally contaminated barley exhibiting gushing potential. *P. anomala* C565 restricted *Fusarium* growth and hydrophobin production during malting and prevented beer gushing. Grain germination was not disturbed by the presence of yeast. Addition of *P. anomala* C565 into the steeping seemed to retard wort filtration, but the filtration performance was recovered when yeast culture was combined with *Lactobacillus plantarum* VTT E-78076. Well-characterized microbial cultures could be used as food-grade biocontrol agents and they offer a natural tool for tailoring of malt properties.

Keywords: malting, yeast, *Fusarium*, gushing factor, biocontrol

INTRODUCTION

The fungal community characteristic to malting barley develops before harvest, during storage and during the malting process. More than 150 species of filamentous fungi and yeasts may be found on cereal grains as surface contaminants or as internal invaders (55). It is well known that barley-derived fungi and their metabolites greatly influence malt and beer quality (17, 43, 67). *Fusarium* moulds are important members of the indigenous fungal community of barley. The abundance of *Fusarium* contamination and the diversity of the species are dictated particularly by crop susceptibility, agricultural practices, climate and geographic location (7, 63). Contamination of the barley crop by fusaria is of concern particularly in years when poor weather conditions favour the growth of toxigenic and gushing-active *Fusarium* species. Gushing is a term used to describe spontaneous overfoaming of beer on opening of the packaged product, and it is often associated with heavy *Fusarium* infection of barley or malt (2, 59). Gushing is a complex phenomenon, which can at least partially be explained by the secretion of specific factors by fungi in barley in the field, during storage, or during the malting process (2, 42, 53). Gushing factors are assumed to be surface active molecules which stabilize CO_2 bubbles in beer by forming a layer around microbubbles (47).

Our recent studies indicated that fungal proteins called hydrophobins act as the gushing factors of beer (26, 30, 53). Hydrophobins are small, secreted, cysteine-

rich proteins (100 ±25 amino acids) that are produced by filamentous fungi (70). Hydrophobins are among the most abundantly produced proteins of fungi and they have various biological roles and unique properties (37). They have the property of self-assembly at hydrophilic-hydrophobic interfaces forming very stable insoluble amphipathic films, and are involved in fungal adherence to surfaces (70). These protein films are commonly found on surfaces of aerial structures such as hyphae, conidia and fruiting bodies (64). A hydrophobic coating has also been proposed to have a protecting role against both dessication and wetting, and to assist spore dispersal. Hydrophobins play key roles in development and in the interactions of fungi with the environment and other organisms, particularly plants (69).

Strict control of incoming barley lots is vitally important in order to reject contaminated material prior to purchasing. However, malting conditions favour the growth of *Fusarium* fungi, including species which might produce mycotoxins and gushing factors during the process (22, 42, 52, 58, 59). Therefore there is a need for efficient and safe ways to control growth and metabolic activity of fungi in raw materials as well as during the processing. Due to current environmental and health concerns, research has been directed towards developing natural means of prevention of fungal grain diseases and spoilage. Biological control with well-characterized, antagonistic microbes or with natural plant- derived and microbial compounds has been introduced into many fields of food and feed processing. The plant-derived microbes, mainly bacteria and yeasts, have shown strong antagonistic activity against various fungal contaminants (38, 45). Biocontrol candidates will most likely persist in the habitat from which they were isolated (16). Starter technology, in which barley is inoculated with well-characterized microbes, has also been introduced to the malting industry (5, 8, 25).

Our previous study revealed that a numerous and diverse yeast community consisting of both ascomycetous and basidiomycetous species was a significant part of the malting ecosystem (35). Several yeasts produced plant cell wall-degrading enzymes with potentially positive contribution to malt processability. It has been reported that several species of ascomycetous and basidiomycetous yeasts of the saprophytic phyllosphere community have strong antagonistic activity against various fungal pathogens (3). Several strains have successfully been applied to prevent pre- and post-harvest fungal diseases of fruits and

vegetables (4, 28) and to control spoilage moulds during the storage of high moisture feed grains (12, 48). However, rather little is known about the antifungal potential of the diverse yeast community occurring in the industrial malting ecosystem. Boivin *et al.* (6) demonstrated that the addition of specific, malt-derived *Geotrichum candidum* (teleomorph *Galactomyces geotrichum*) into the malting process restricted fungal growth and prevented mycotoxin formation. This application has been developed into commercial scale.

Biocontrol strains are often introduced to various applications as single cultures. Recently research has also been directed to combining several biocontrol agents or linking microbial cultures with other preservation methods (68). Yeast and lactic acid bacteria often occur together in plant-based bioprocesses and synergistic interactions between these two groups are utilized in many cereal fermentations (4). We have previously shown that the addition of lactic acid bacteria (LAB) into malting activated the indigenous yeast community and enhanced production of microbial β-glucanase and xylanase in the malting process (34). Combining antagonistic yeast with lactic acid bacteria might further enhance the usefulness of starter technology in complex bioprocesses such as malting.

The present study was designed to elucidate the antifungal potential of yeasts isolated from industrial maltings. Furthermore, the effects of a selected strain, *Pichia anomala* VTT C-04565 (C565) were investigated in a true malting environment with naturally infested barley showing gushing potential. The ultimate goal was to suppress *Fusarium* growth and to prevent the production of gushing-inducing hydrophobins during malting. We also studied the effects of *P. anomala* C565 in combination with *Lactobacillus plantarum* VTT E-78076 starter culture in order to enhance malt processability.

MATERIALS AND METHODS

Fungal cultures

The yeast cultures, including 7 ascomycetous and 5 basidiomycetous strains, and 21 filamentous fungi were provided by the VTT Culture Collection (Table 1). The yeasts were chosen on the grounds that they occur spontaneously in the

malting ecosystem (35). Furthermore, strains belonging to the species *A. pullulans, C. sake, C. saitoana, Cr. albidus, G. geotrichum, P. anomala* and *P. guilliermondii* had also shown antifungal potential in other plant applications (4, 6, 44, 71). In addition, 4 basidiomycetous yeasts (*Cr. albidosimilis*, *Cr. curvatus*, *Cr. magnus*, *R. pinicola*) were tested as they were shown to produce plant cell wall-degrading enzymes (35). Yeast strains were grown on yeast-malt extract agar, YM-agar (Difco Laboratories, Detroit, MI, USA) at 25 °C for 2–3 days. The yeast cultures were stored in 10% glycerol at -70 °C for long-term storage, and on YM-slants at 4 °C for short-term storage. The filamentous fungi originated from barley and malted barley samples. The mould cultures were grown at 25 °C for 7 days on Potato Dextrose Agar (PDA, Difco) and maintained on PDA-slants at 4 °C.

Antifungal screening *in vitro* with plate-assay

The yeast strains were screened for antifungal potential using a dual-culture overlay assay adapted from Magnusson *et al.* (39). Yeasts were inoculated along a 2 cm line on replicate YM plates and allowed to grow at 25 °C for 2–3 days. The plates were overlaid with 10 ml of temperated malt extract soft agar (0.05% malt extract, Difco) containing 10^4 fungal spores per ml. Spore suspension was prepared by removing the spores from the PDA plates of a 7 day-culture. Sterile saline (10 ml) was added to the plates and spores were harvested with a bacteriological spreader. The suspension was filtered through sterile glass wool to remove mycelial debris. The number of spores was counted microscopically using a counting chamber (Thoma, Knittle Gläser, Germany) and adjusted by adding sterile distilled water. The growth inhibition was measured after 5 and 7 days of incubation at 25 °C. The results were considered as positive (+) if the mould could not overgrow the yeast or if a clear inhibition area was observed around the colony. In the negative (-) samples the whole plate was covered with mycelia.

Malting experiments with naturally infested barley

Barley (*Hordeum vulgare* L, Poaceae, two-row variety Scarlett cultivated in Finland 2005) samples (1 kg) were malted in a specially designed, computer-controlled micro-malting equipment with a separate drum for each sample (Hulo Engineering, Helsinki, Finland). Due to the intensive *Fusarium* contamination and gushing potential this sample was unacceptable for commercial purposes,

but suitable for this study. Fungal gushing factors were not found in the native barley, but were produced during the malting process. Before malting, barley samples were sieved to remove grains <2.5 mm. All barley samples were steeped in 3 L of water or in water containing microbial cultures at 18 °C for 8 h, followed by a 16 h air rest (20 °C) and a second steep (2 h, 18 °C). The moisture content of grains was measured daily and kept constant (46–47%) by adding water. The barley was then allowed to germinate for 5 days at 16 °C and dried (kilned) in warm air (4 h 50 °C, 3 h ramp to 60 °C, 2.5 h 60 °C, 3 h ramp to 85°C, 1 h 85°C) in a separate kiln. The rootlets were removed before analyses.

Liquid cultures of *Pichia anomala* C565 strain were grown in Erlenmayer flasks containing YM-broth and incubated on a rotary shaker at 100 rpm at 25 °C for 3 days. Cells were harvested by centrifugation at 5300 g for 10 min. Cell counts were determined microscopically using a Thoma counting chamber and adjusted to the desired level with sterile distilled water. Three individual malting experiments were carried out with a pure culture of *P. anomala* C565. In experiment 1 (Exp. 1) yeast cultures were added into the first steeping water at a level of 10^6cfu/ml. In experiments 2 (Exp. 2) and 3 (Exp. 3) *P. anomala* C565 was added into both steeping waters (10^6 cfu/ml). In malting experiment 4 (Exp. 4) *P. anomala* C565 was combined with *Lactobacillus plantarum* VTT E-78076 (E76) in duplicate samples. *L. plantarum* E76 strain was grown in MRS-broth (Oxoid) at 30 °C for 3 days. LAB culture, including cells and spent medium, was added into the first steeping water at a level of 4% v/v of the steeping water. The LAB were enumerated on MRS agar plates (Oxoid) incubated in anaerobic conditions at 30 °C for 72h. *P. anomala* C565 cells were added into the second steeping water.

The number of germinated grains was counted daily from a sample of about 150–200 kernels until the germination rate exceeded 90%. The concentration of ethanol in the head space of each malting drum was analyzed continuously using a Fourier Transform Infrared Spectroscopy (FTIR) multicomponent gas analyzer Gasmet® (Temet Instruments ltd, Helsinki, Finland) with a heated, flow-through, 5 m path length sample cell.

Microbiological analyses of process samples

Samples for the microbiological analyses were taken from untreated barley, and from barley after steeping, germination and kilning (after rootlet removal). The

following microbial groups were analyzed from homogenized barley samples: aerobic heterotrophic bacteria, *Pseudomonas* spp., lactic acid bacteria, and yeasts. Duplicate samples were prepared in each experiment. A sample of 10 g was homogenized for 10 min with 90 ml of sterile saline in a Stomacher Lab Blender 400 (Seward Medical, London, UK). Aerobic heterotrophic bacteria were determined on plate count agar (PCA, Difco Laboratories, Detroit, USA) and *Pseudomonas* spp. on C-F-C agar (Oxoid Ltd., Basingstoke, Hampshire, UK). Samples were incubated in aerobic conditions at 30 °C for 2–3 days. The number of LAB was determined on MRS agar (Oxoid) and samples were incubated in anaerobic conditions at 30 °C for 5 days. To prevent fungal overgrowth of bacterial determinations, 0.001% cycloheximide (Sigma Chemical, St. Louis, MO, USA) was added to PCA, C-F-C and MRS media. Yeast counts were determined on YM agar (Difco Laboratories). Samples were incubated in aerobic conditions at 25 °C for 3–5 days. Chlortetracycline and chloramphenicol (both at 0.01%) were added to YM medium to prevent bacterial growth. In addition, 0.02% of Triton-X 100 (BDH) was used to limit the spreading of fungal colonies on YM-agar. The bacteria and yeast results are expressed as colony forming units/gram barley (cfu/g).

For *Fusarium* analyses 100 randomly selected kernels were placed on a selective Czapek-Dox agar containing Iprodion and Dichloral (CZID-agar) (1, 15). The CZID plates were incubated at 25 °C for 7 d. Other filamentous fungi such as *Alternaria* spp., *Cephalosporium* spp., *Cladosporium* spp., *Drechslera* spp., *Epicoccum* spp., *Mucor* and *Rhizopus* spp. were determined from barley, steeped barley and malt samples on wet filter paper using direct plating of 100 kernels (15). Filter paper plates were incubated at 25 °C for 21 d. Fungi were identified under a stereomicroscope on the basis of typical colony form and colour. Identification was confirmed by conidia morphology with a light microscope (magnification 400x). The results are expressed as per cent of kernels contaminated with fungi.

Determination of fungal hydrophobins and gushing potential

The hydrophobin levels in the malt samples were determined with competetive ELISA (Enzyme Linked ImmunoSorben Assay) as described by Sarlin *et al.* (53). Ground sample (5g) was extracted with PBS buffer (10 mM sodium phosphate pH 7.3, 150 mM sodium chloride) in the proportion of 1:10. After

centrifugation the supernatant was removed to a clean tube and antibodies against *F. poae* VTT D-82182 (D182) were added. After incubation, the sample-antibody mixture was transferred to an immunoplate (Nunc-Immuno Modules, MaxiSorp polystyrene strips, Nunc, Rochester, USA) coated with hydrophobin extract of *F. poae* D182 Goat anti-rabbit IgG (H+L)-alkaline phosphatase (AP) conjugate (Bio-Rad Laboratories) was used as a secondary antibody. p-Nitrophenyl phosphate tablets (Sigma, St. Louis, USA) in diethanolamine-$MgCl_2$ buffer (Oy Reagena Ltd, Toivala, Finland) were used as substrate for AP detection. The absorbance was read at 405 nm. Due to the nature of the competitive ELISA, a lower absorbance value corresponded to a higher amount of hydrophobin in the samples. In the present study, the results are expressed as the inverse of the mean absorbance value. The results are means of the analyses of four replicates. The significance of *P. anomala* C565 for malt hydrophobin levels in three malting experiment was evaluated using one-way variance analysis (ANOVA) with Tukey's Honestly Significant Difference (HSD) test. Statistical significance was assessed at $P<0.05$. The software SPSS 14.0 for Windows was used for the statistical analyses.

The gushing potential of malt samples was measured as described by Vaag *et al.* (66). The malt extracts were added to commercial, bottled beer (0.33l) and pasteurized bottles were agitated for three days with a horizontally rotating shaker at 50 rpm (21). The gushing positive and negative malt samples obtained from Carlsberg Research Laboratory (Denmark) were included in the studies. After shaking, the bottles were kept still for 10 min, inverted three times and opened after 30 seconds. The amount of gushing was determined from the change in weight of the bottle. The test was performed in triplicate.

Malt and wort analyses

High gravity mashing and the Büchner filtration test for evaluation of lautering performance were performed as described by Sjöholm *et al.* (61). The high gravity laboratory mashing conditions resemble those used in commercial brewery practice, so the results give a better prediction of the brewing performance of malt than the standard EBC Congress mash. Samples were analyzed using the following EBC recommended methods: malt friability, wort extract content, wort soluble nitrogen, wort free amino nitrogen, wort viscosity and wort β-glucan (14). α-Amylase activity was analyzed with a Ceralpha kit

(Megazyme International Ireland Ltd, Wicklow, Ireland) using an extraction time of 30 min and assay conditions as specified by the manufacturer. β-Glucanase activity was analyzed with the Azo-barley glucan method kit using azo-barley glucan as substrate (Megazyme). The assay was performed at both 30 and 60 °C in order to distinguish between β-glucanase of barley and microbial origin. Xylanase was analysed with an *endo*-1,4-β-xylanase assay procedure using Xylazyme AX tablets (Megazyme) as substrate. Milled malt (1.00 g) was extracted in 8.0 ml of sodium acetate buffer (25 mM, pH 4.5) for 15 minutes at room temperature with continuous stirring (200 rpm). The flour was separated by centrifugation (1000 g). Xylanase activity was measured at 45 °C. A substrate tablet was added to 0.5 ml of extract and incubated for 30 minutes. The reaction was stopped by adding 5.0 ml of 1% Trizma base. Absorbance was measured at 590 nm. The results are expressed as difference in absorbance between the sample and a reagent blank.

RESULTS

Antifungal potential of yeasts and yeast-like fungi against filamentous field and storage fungi

The antifungal potential of 7 ascomycetous and 5 basidiomycetous yeast strains was screened against common field and storage contaminants using a dual-culture plate assay, in which yeast cultures were first grown in YM-agar and then overlayed with mould spore suspension in soft malt agar (Table 1). The main emphasis of this study was on the suppression of *Fusarium* growth, and therefore 13 different *Fusarium* strains were tested. The results indicated that the ascomycetous yeasts had better antifungal potential than the basidiomycetous yeasts. As seen from Table 1, *C. saitoana* C524, *Geotrichum s*p. D559, *P. anomala* C564 and C565, and *P. guilliermondii* C568 were the most prominent strains with respect to antagonistic activity against filamentous fungi. When grown together on solid media, these yeasts clearly suppressed the growth of several indicator moulds. However, great variation in growth inhibition was observed between different mould species and even between strains. All the yeast strains tested could prevent the overgrowth of *F. avenaceum* D141 in the plate assay, whereas *F. oxysporum* D134 and *F. tricinctum* D607 strains were inhibited only by *Geotrichum* sp. D559, *P. anomala* C564 and C565.

Table 1. Antifungal potential of various yeasts and yeast-like fungi isolated from an industrial malting ecosystem against field and storage moulds in a plate-screening assay.

Fungal strain		**Ascomycetous yeasts and yeast-like fungi**							**Basidiomycetous yeasts**				
	VTT-D	*Aureobasidium pullulans* D-04**1014**	*Candida sake* C-95**520**	*C. saitoana* C-04**524**	*Geotrichum* sp. D-04**559**	*Pichia anomala* C-04**564**	*P. anomala* C-04**565**	*P. guilliermondii* C-04**568**	*Cryptococcus albidus* C-92**012**	*Cr. albidosimilis* C-04**508**	*Cr. curvatus* C-04**536**	*Cr. magnus* C-04**540**	*Rhodotorula pinicola* C-04**571**
Achremonium polychronum	96**653**	-	-	+	+	+	+	+	-	-	-	-	-
Alternaria alternata	76**024**	+	-	-	+	+	+	-	-	-	-	-	-
Aspergillus ochraceus	00**808**	-	-	+	+	+	+	-	-	-	+	-	-
Cochliobolus sativus	76**039**	+	-	+	+	+	+	+	-	-	+	+	-
Eurotium amstelodami	03**923**	+	+	+	+	+	+	+	+	+	+	-	-
Fusarium avenaceum	80**141**	+	+	+	+	+	+	+	+	+	+	+	+
F. cerealis	96**601**	+	+	+	+	+	+	+	-	+	+	-	-
F. culmorum	80**148**	-	-	+	+	+	+	+	-	-	-	-	-
F. equiseti	82**087**	-	+	+	+	-	+	-	-	-	-	-	-
F. graminearum	82**169**	-	-	+	+	+	+	+	-	-	-	-	-
F. graminearum	95**470**	+	-	+	+	+	+	+	-	+	-	-	-
F. langsethiae	03**931**	-	-	+	+	+	+	-	-	-	-	-	-
F. oxysporum	80**134**	-	-	-	+	+	+	-	-	-	-	-	-
F. poae	76**038**	-	-	+	-	+	+	+	-	-	-	-	-
F. sambucinum	77**056**	-	-	+	+	+	+	-	-	-	-	-	-
F. sporotrichioides	82**175**	-	-	-	+	+	+	+	-	-	-	-	-
F. sporotrichioides	72**014**	+	-	+	+	+	+	-	+	-	-	-	-
F. tricinctum	96**607**	-	-	-	+	+	+	-	-	-	-	-	-
Penicillium verrucosum	01**847**	+	-	+	+	+	+	+	-	+	-	-	-
P. verrucosum	00**831**	+	-	+	+	+	+	+	-	-	-	-	-
Pyrenophora teres	89**395**	+	+	+	+	+	+	+			-	-	-

- no inhibition, + suppression of mould growth

Antimicrobial effects of *P. anomala* C565 in malting of naturally infested barley

P. anomala C565 was selected for the malting experiments because it suppressed the growth of all indicator organisms in the *in vitro* study. Three individual malting experiments were carried out with *P. anomala* C565 single strain culture. In Exp. 1, the yeast culture was added into the first steeping water, and in Exp. 2 and 3 into both steeping waters. The cells were added into the steeping waters at a level of 10^6 cfu/g of barley. As seen from Fig. 1., the counts of *P. anomala* C565 increased over 1 log unit during the first days of malting and reached their maxima ($3x10^8$ cfu/g) at the end of germination. Kilning had little effect on the viable counts. The yeast counts in the final malt were over 10^6 cfu/g in Exp. 1 and over 10^7 cfu/g in Exp. 2 and 3. *P. anomala* C565 suppressed the growth of other yeasts on the YM-plates and only *P. anomala* colonies were detected, whereas a diverse yeast population was detected in the control samples.

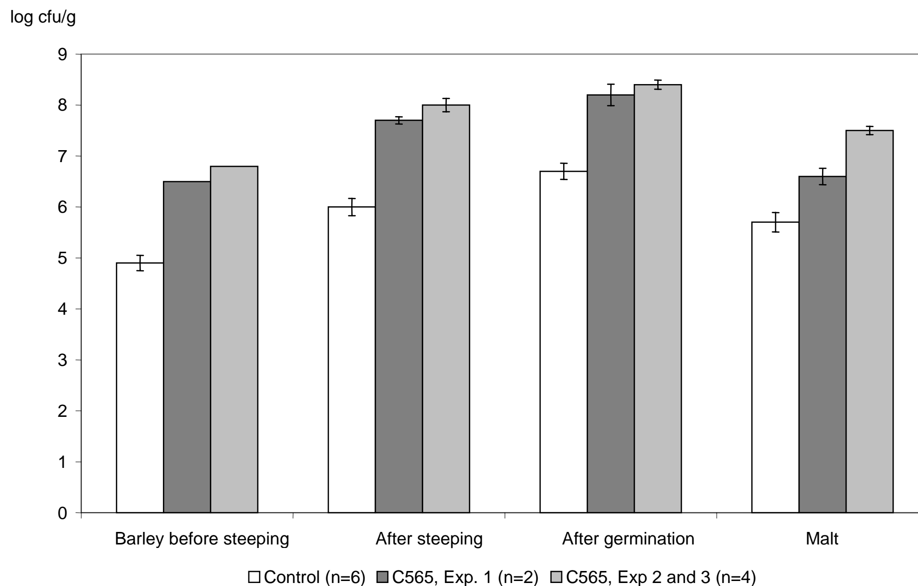

Figure 1. Growth of indigenous yeasts (control) and inoculated P. anomala yeasts (C565) during the malting experiments. Duplicate grain samples were determined in each experiment. The control counts are mean values obtained from 3 individual malting experiments (± standard deviation). P. anomala C565 counts are averages of duplicate samples from Exp.1 (C565 added into the first steeping water) and averages of four samples from Exp. 2 and 3 (C565 added into both steeping waters).

Many antifungal studies have been carried out in controlled laboratory environments with pure cultures or with artificially contaminated material. In this study the antifungal potential of malt-derived yeast was evaluated with naturally contaminated material showing gushing potential. The addition of *P. anomala* C565 into the steeping water clearly suppressed the intensity of *Fusarium* contamination and obviously modified the *Fusarium* population (Fig. 2). Although the CZID-analysis showed that 100% of the kernels were contaminated after steeping, a clear visual difference in *Fusarium* populations was observed between the control (Fig. 2A) and *P. anomala* C565 treated samples (Fig. 2B) after steeping. The *Fusarium* contamination in the final malt samples remained high (99% of the kernels were contaminated with fusaria). Only approximately 7% lower counts were measured in the malt samples after *P. anomala* C565 treatment. Direct plating method with CZID-agar had limited quantitative value and indicated only the fraction of kernels contaminated with fungi, not the degree of infection. Therefore, the effects of *P. anomala* C565 on *Fusarium* fungi were also evaluated indirectly by determination of fungal hydrophobins, also known as gushing inducers.

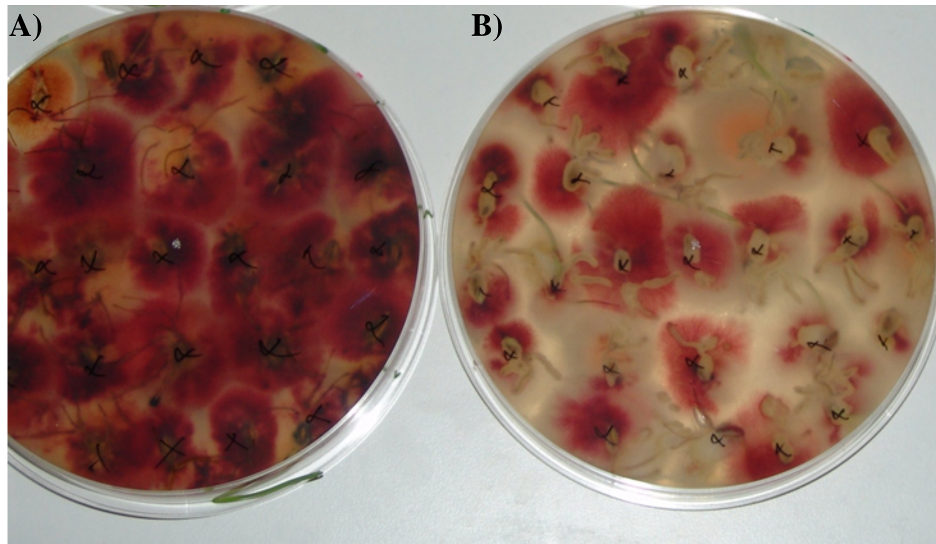

Figure 2. Fusarium growth restriction by P. anomala C565 added to the steeping water. Kernels contaminated with Fusarium fungi after 2 days of malting (after steeping) on CZID plates. A) control sample, B) P. anomala C565 added to both steeping waters.

The results of the hydrophobin-ELISA revealed that the addition of *P. anomala* C565 clearly restricted the production of *Fusarium* hydrophobins during malting (Fig. 3). Analysis of variance showed that the malt hydrophobin levels of the control and *Pichia*-treated samples were significantly ($P<0.05$) different. As also can be seen from Fig. 3, the hydrophobin levels in the control samples of Exp. 1 differed significantly ($P<0.05$) from those of Exp. 2 and 3. The first malting experiment was carried out with barley after six months of storage and the subsequent experiments with the same barley sample after 7 and 7.5 months of storage. The results indicated that the hydrophobin formation capability of fusaria was reduced during the prolonged barley storage.

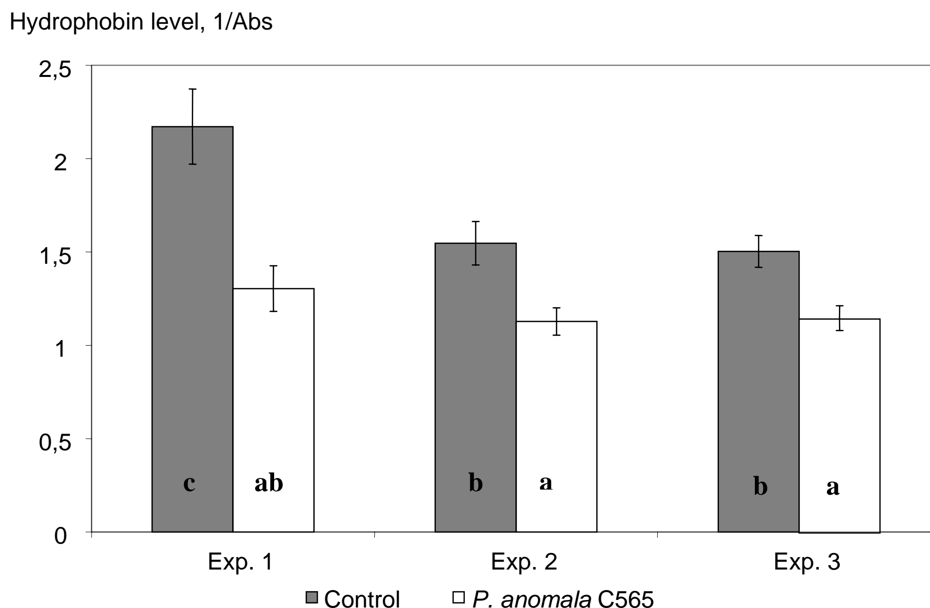

Figure 3. Effects of P. anomala C565 on malt hydrophobin levels. P. anomala C565 was added into the first steeping water in Exp. 1 and to both the first and second steeping water in Exp. 2 and 3. Values are means of four replicates (±standard deviation). Bars labelled with different letters are statistically different at the significance level of 0.05.

The gushing test confirmed that beer gushing was prevented when *P. anomala* C565 was added into the steeping waters of barley. All the control samples induced overfoaming of beer whereas gushing tendency was not observed in the *P. anomala* treated samples (Table 2).

Table 2. Effects of P. anomala C565 on malt gushing potential. P. anomala C565 was added into the first steeping water in Exp. 1 and to both first and second second steeping water in Exp. 2 and 3.

	Gushing tendency [a)]	
Experiment	**Control**	***Pichia anomala* C565**
Exp. 1	17 ± 16	0 ± 0
Exp. 2	32 ± 5	0 ± 0
Exp. 3	1 ± 1	0 ± 0

[a)] Gushing of beer was determined as the beer overflowing (g) from the bottles. The test was performed in triplicate.

We also studied the effect of *P. anomala* C565 addition on the growth of other filamentous fungi during malting. As seen from Fig. 4, the common field fungi *Alternaria, Cephalosporium*, *Cladosporium* and *Drechslera,* were not restricted by the addition of *P. anomala* C565. On the contrary, slightly higher (10%) *Drechslera* and *Cephalosporium* counts were observed after steeping of *P. anomala* C565 treated samples compared to the control. The *Mucorales* fungi, such as *Mucor* or *Rhizopus*, did not belong to the indigenous fungal community of barley (Fig. 4). They are commonly detected as process contaminants at elevated moisture conditions, especially during the early hours of kilning. The fungal analysis of malt samples revealed that over 80% of the control kernels were contaminated with *Mucor* fungi. A significant reduction of this fungus was measured in *P. anomala* C565 treated samples. Only 26% of the malt kernels contained *Mucor* fungi after *P. anomala* treatment.

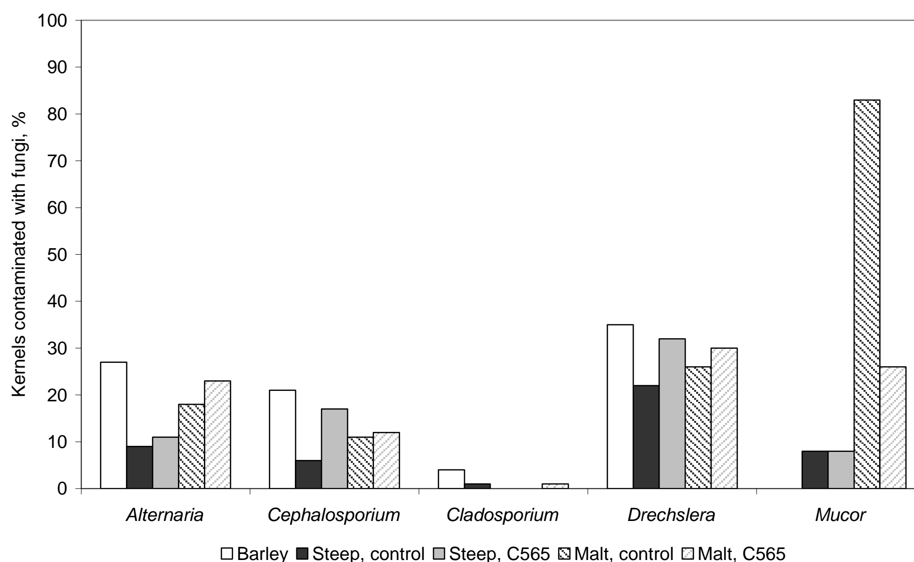

Figure 4. Effects of Pichia anomala C565 added to the first and second steeping water on the occurrence of Alternaria, Cephalosporium, Cladosporium, Drechslera and Mucor fungi in barley samples after steeping and in final malt. The values are means of the two malting experiments (Exp. 2 and 3).

Addition of *P. anomala* C565 as single culture into the steeping waters had no effect on the bacterial community consisting of both Gram-negative and -positive bacteria. The aerobic bacterial count reached 10^9 cfu/g after 5 days of germination in both control and *Pichia*-treated samples. In the final malt samples after rootlet removal, the number of aerobic heterotrophic bacteria was 10^8 cfu/g. A significant proportion of this aerobic bacterial population was composed of pseudomonads (10^6 cfu/g). The indigenous LAB population was low in barley, but increased considerably during malting in both control and *P. anomala* C565 samples. The final malt contained 10^7 LAB/g.

Effects of *P. anomala* C565 on grain germination and malt quality

P. anomala C565 had no notable effect on grain germination (Table 3). Over 96% of the kernels had germinated in both samples after 3 days of malting. Interestingly, *P. anomala* C565 clearly decreased the ethanol concentration in the head space of a malting drum (Fig. 5). After carbon dioxide, ethanol was the second most abundant volatile detected in the control samples. The present results

indicate that the ethanol produced by the grain was rapidly consumed by *P. anomala*. The composition of the gas atmosphere in the malting drum also differed with respect to ethyl acetate, which was only detected in *Pichia*-inoculated samples, at low levels (1.5–7 ppm) during the first two days of malting.

Table 3. Effects of P. anomala C565 addition on grain germination. The values are means ± standard deviation of three individual malting experiments (Exp. 1–3).

	Germinated grains, %	
Malting time, day	**Control**	***Pichia anomala* C565**
1	16 ± 1	11 ± 4
2	79 ± 2	71 ± 4
3	97 ± 3	96 ± 1

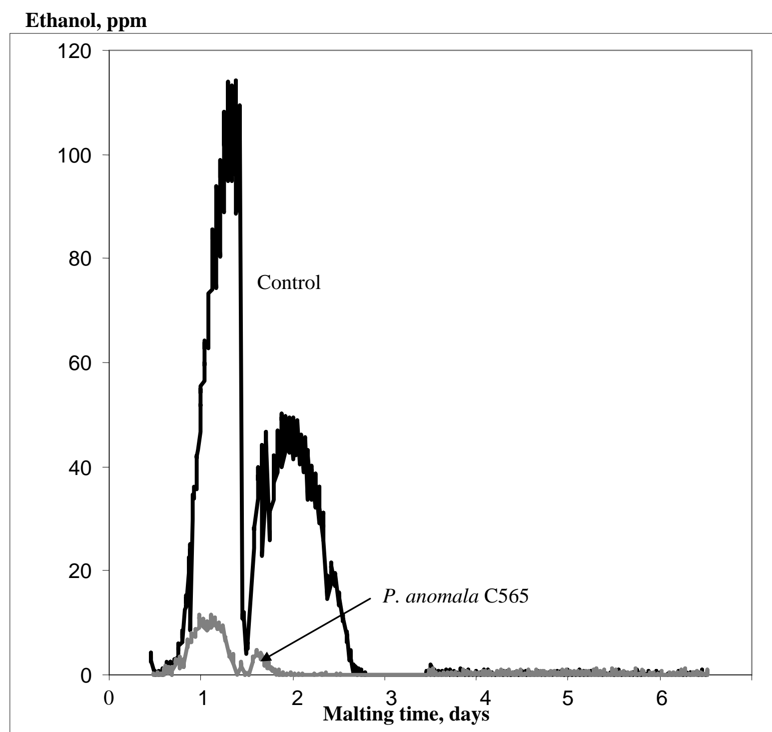

Figure 5. Effects of P. anomala C565 on ethanol production during the first days of malting. The results represent one of the duplicate malting experiments (Exp. 2).

Table 4 shows the effects of *P. anomala* C565 on malt quality and on the properties of high gravity wort. The malts were all well modified based on the high friabilities. However, this study indicated that *P. anomala* C565 addition may retard mash filterability (Fig. 6A). The difference between the filtration curves of the control and *Pichia* treated samples was small but consistent. Approximately 10% less filtrate was obtained within one hour of *Pichia* treated samples (when added into both steeping waters) compared to control samples. As seen from Table 4, *P. anomala* appeared to suppress the microbial β-glucanase (assayed at 60 °C) and xylanase activities in malt in the Experiments 2 and 3, which could partly explain the impeded wort filtration.

Table 4. Effects of P. anomala C565 on malt and wort (High gravity) properties. P. anomala C565 was added into the first steeping water in Exp. 1 and to both the first and second second steeping water in Exp. 2 and 3.

	Control [a]	***P. anomala* C565**	
	Mean n = 3	Exp. 1 n = 1	Exp. 2 and 3 n = 2
Malt analyses			
Friability, %	88 ± 2	88	86
α-amylase, U/g	320 ± 6	352	321
β-glucanase, 30 °C, U/kg	722 ± 46	720	684
β-glucanase, 60 °C, U/kg	114 ± 15	94	105
Xylanase, abs x 1000	0.209 ± 0.05	0.213	0.184
High gravity wort analyses			
Wort extract content, w-%	16.8 ± 0.1	17.1	16.7
Colour, EBC	5.7 ± 0.2	5.5	5.6
Free amino nitrogen, mg/l	367 ± 9	404	366
Soluble nitrogen, mg/l	1819 ± 14	1891	1807
pH	5.6 ± 0	5.6	5.5
β-glucan, mg/l	263 ± 15	260	270
Wort viscosity, cP	2.17 ± 0.03	2.18	2.26

[a] the values for control samples are means ± standard deviation of three individual malting experiments (Exp. 1–3).

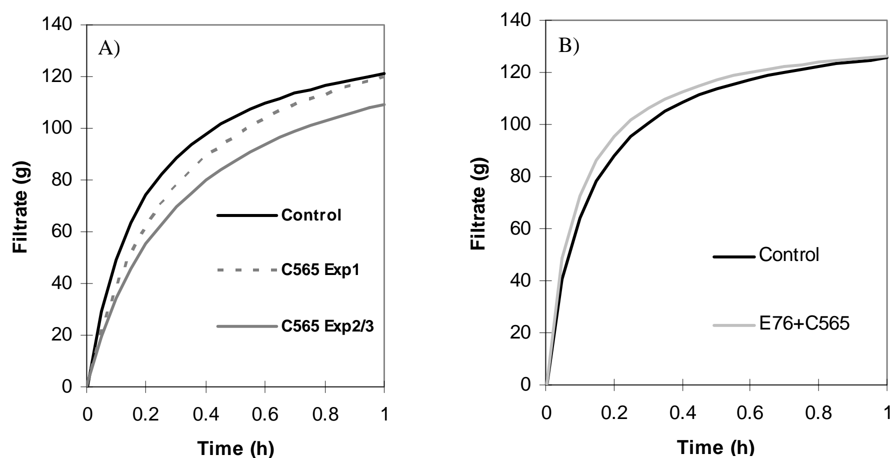

Figure 6. Effects of P. anomala C565 (A) and of a combination of P. anomala C565 with L. plantarum E76 (B) on mash filterability measured as the Büchner filtration test. Values are means of triplicate (Control in Fig. A), duplicate (C565 Exp2/3 in Fig. A; E76+C565 B) or single (C565 Exp1 in Fig. A; Control in Fig. B) malting samples. The repeatability of the filtration curve has been evaluated by including standard malt in each analysis during several years. In the standard malt, the standard deviation of the amount of filtrate measured at 0.25 h is 5.1 g.

Combination of *P. anomala* C565 with *Lactobacillus plantarum* E76 starter culture

In order to improve the wort filtration performance, *P. anomala* C565 was combined with *L. plantarum* E76 in Exp. 4. *L. plantarum* E76 was added to the first steeping water and *P. anomala* C565 to the second steeping water. As seen from Fig. 6B, the filtration performance was recovered when these two treatments were combined. *L. plantarum* E76 treatment enhanced the production of plant cell wall hydrolysing enzymes of microbial origin: slightly higher microbial β-glucanase activities relative to the control were observed when *L. plantarum* E76 was combined with *P. anomala* C565 (Table 5). In addition, part of the beneficial effects obtained with lactic acid starter treatment can be explained by reduced growth of gram-negative bacteria, particularly pseudomonads (Fig. 7) with a negative influence on mash filterability.

Table 5. Effects of combined treatment with L. plantarum E76 (added to first steeping water) and P. anomala C565 (added to second steeping water) on malt and wort (High gravity) properties.

	Control n = 1	***L. plantarum* E76 + *P. anomala* C565** n = 2
Malt analyses		
Friability, %	86	84
α-amylase, U/g	352	356
β-glucanase, 30 °C, U/kg	673	638
β-glucanase, 60 °C, U/kg	94	166
Xylanase, abs x 1000	0.203	0.278
High gravity wort analyses		
Wort extract content, w-%	16.6	16.6
Colour, EBC	6.0	7.0
Free amino nitrogen, mg/l	356	392
Soluble nitrogen, mg/l	1809	1882
pH	5.5	5.5
β-glucan, mg/l	210	205
Wort viscosity, cP	2.12	2.07
Gushing tendency [a)]	23 ± 17	0 ± 0

[a)] Gushing of beer was determined as the beer overflowing (g) from the bottles. The test was performed in triplicate.

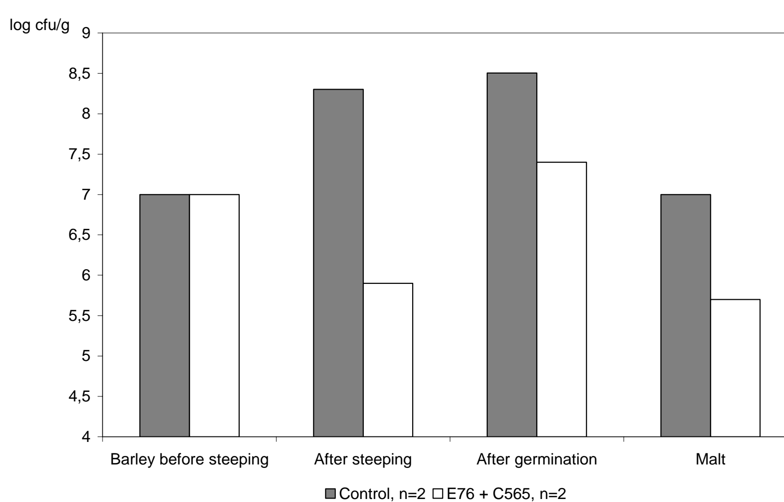

Figure 7. Effects of L. plantarum E76 and P. anomala C565 combination on the growth of Pseudomonas spp. during malting. Values are means of duplicate malting samples.

DISCUSSION

Malting can be considered as a complex ecosystem consisting of germinating grain and a complex microbial community including a number of aerobic bacteria, lactic acid bacteria, yeasts and filamentous fungi (35, 43, 50). It is obvious that microbes greatly influence malt quality, wort filtration and fermentation and therefore have a significant impact on beer processing and quality. Depending on the nature and extent of microbes the effects may be either beneficial or deleterious to malt quality (6, 10, 17, 23, 33, 38, 52, 60, 65, 67). The most negative consequences linked to intensive mould growth, especially fusaria, are the production of mycotoxins and gushing factors (53, 54, 58).

This study indicated that *Fusarium* growth during malting and the production of fungal hydrophobic proteins, also known as gushing factors, could be suppressed with yeasts naturally occurring in the industrial malting ecosystem. *In vitro* screening with a plate-assay indicated that ascomycetous strains belonging to species of *A. pullulans*, *C. sake, C. saitoana*, *G. geotrichum*, *P. anomala*, and *P. guilliermondii* were the most potential yeasts with respect to antifungal activity. These results were in agreement with previous investigations (6, 19, 46, 51, 56, 72). *P. anomala* VTT C-04565 (C565) was selected for malting experiments in order to verify the antifungal potential of malt-derived yeast in malting with naturally infested barley. To our knowledge this is the first report showing the effects of *P. anomala* against *Fusarium*-fungi in malting and on overall malt quality.

P. anomala is a robust organism which is occurring naturally in plant materials such as in cereals (45). It is traditionally used in fermented products in Africa and Asia (44). This species is classified as safe (biosafety level 1), and is potentially a suitable biocontrol agent in a variable environment (12, 15, 45). *P. anomala* has previously shown antimicrobial activity against a wide range of unrelated microbes such as bacteria, yeasts, and filamentous fungi (45). *P. anomala* J121 has been extensively studied in the preservation of moist grains (wheat, barley and oats) for animal feed (12, 18, 19, 48, 49).

We demonstrated that *Pichia anomala* C565 added to the steeping water restricted *Fusarium* growth. Steeping can be regarded as the most important step in malting with respect to microbiological safety because it activates rapid

growth of bacteria and fungi (43). Therefore, *P. anomala* C565 was inoculated at this stage. Although direct plating had little quantitative value in *Fusarium* biomass evaluation, clear suppression of *Fusarium* growth was observed on grains cultivated on CZID-agar. Apparently the majority of the *Fusarium* community was located in and on the outermost layers of barley tissues and was therefore restricted by the addition of *P. anomala* C565. In addition to *Fusarium* inhibition, *P. anomala* C565 treatment restricted *Mucor*-contamination. Mucrorales fungi, such as *Mucor* and *Rhizopus,* are considered as surface contaminants of grains and they proliferate during germination and the early stages of kilning (11). This finding also suggested that yeasts may suppress the attachment of fungal surface contaminants.

However, *P. anomala* did not totally inhibit fusaria. Moreover, the growth of other field fungi was not inhibited by the *P. anomala* addition into the malting process, although inhibition of several filamentous fungi was observed in *in vitro* screening with a plate assay. On the contrary, suppression of *Fusarium* growth most probably provided more nutrients and space for the growth of certain other fungi such as *Cephalosporium* and *Drechslera*. This finding supports the theory that some species were located deeper in the husk layers and were not necessarily influenced by the external addition of biocontrol agent. The field fungi occur in different parts of the husk and pericarp layers in barley (57). Therefore, this study highlights the importance of verification of the results obtained from *in vitro* studies with pure cultures by using naturally infested material *in vivo*. Furthermore, the plate-screening assay indicated that differences in sensitivity might occur between *Fusarium* species and even between strains. However, *Fusarium* diversity after *Pichia* treatment was not analysed in this study, and therefore we cannot conclude which specific species were inhibited during malting. In Finland, the most common *Fusarium* species in barley during the recent years have been *F. avenaceum, F. athrosporioides, F. sporotrichioides* and *F. culmorum* (74). Our further studies will be directed to investigating the effect of biocontrol yeasts on *Fusarium* diversity during processing.

This study also indicated that *P. anomala* C565 suppressed the production of fungal hydrophobic proteins during malting. Hydrophobins are among the most important structural proteins found in the filamentous fungi (13). They are produced in response to changes in the environment and they react to interfaces

between fungal cell walls and the air or between fungal cell walls and solid surfaces (29). We recently showed that fungal hydrophobins are also involved in beer gushing (53). Addition of *P. anomala* C565 into steeping prevented beer gushing. Results obtained with the novel competitive hydrophobin- ELISA test showed that all the *P. anomala* C565 malt samples had absorbance values >0.8. Sarlin *et al.* (53) reported that the risk of gushing is increased if the absorbance value of malt is <0.6. The production of gushing factors in barley and in malting is complex and still a largely unknown phenomenon. It is well known that intensive *Fusarium* growth is part of the normal malting process. However, the production of gushing factors occurs only rarely. Our results suggested that some suppression probably occurs in normal industrial practice as a result of indigenous yeasts.

It has been shown that gushing potential can be decreased during steeping, indicating that part of the gushing factors produced during the growth period of barley in the field are washed away with the steeping waters (42, 52). However, additional hydrophobin production may occur again during germination. Production of hydrophobins is most probably linked to variable environmental conditions and attachment of fusaria to barley surfaces. Gjertsen (20) speculated that the gushing factors were produced as a result of interactions between the barley and fungal mycelium. Munar & Sebree (41) also reported that an extract of *Fusarium* fungi grown on agar plates did not induce gushing when spiked into beer, although when *Fusarium* was grown together with barley, beer gushing occurred. These studies suggest that gushing factors arise as a result of an interaction involving viable mould and the germinating grain. Hydrophobin production may also be species related. Gushing factors formed during malting occurred under the barley husk and could not be removed by washing of the final malt (42). Therefore, preventive actions are essential in assuring safety along the barley to beer chain.

The antifungal action of biocontrol yeasts is often due to several antagonistic mechanisms and hitherto no single mechanism has been shown to be responsible for the whole antimicrobial action. The mechanisms are poorly understood, especially in complex ecosystems. Although the mechanisms in the malting ecosystem were not studied in the present investigation and remain to be revealed, our results indicated that *P. anomala* C565 competed with fusaria for space. As a fast-growing organism, *P. anomala* colonized the outer layers of

barley and suppressed the adherence of fungal contaminants to barley surfaces. Competition for nutrients and space has often been suggested as the main mode of the action mechanism of several biocontrol agents. In addition, the antifungal action of biocontrol yeasts often includes induction of the plant defence system, mycoparatism, production of lytic enzymes such as β,1-3 glucanase or chitinase that degrade the fungal cell wall or secretion of antimicrobial compounds such as killer proteins (28, 40, 45). Druvefors *et al.* (12) suggested that the antifungal effect of *P. anomala* was probably due to the synergistic effect of ethyl acetate and ethanol produced by *Pichia* in an oxygen limited environment. Ethyl acetate was indeed detected in the gaseous atmosphere of the malting drums in *P. anomala* treated samples.

In this study *P. anomala* C565 rapidly consumed the ethanol produced by the grains during the air rest. *P. anomala* can utilize ethanol as a growth substrate in aerobic conditions (32, 62). We recently reported that the ethanol detected during the first days of malting was mainly produced by the barley embryo and the aleurone cells (71). Fermentative metabolism and concomitant ethanol production is part of the normal grain germination. *Pichia* yeasts can utilize a wide variety of carbon and nitrogen sources for growth. Our results suggested that *P. anomala* can utilize the grain metabolites as substrate for growth, without disturbing the grain germination process.

This study confirmed previous findings that *P. anomala* had great antifungal potential (12, 19, 48, 49), and expanded the list of potential application areas. However, there seemed to be a trend towards slightly lower wort separation when *P. anomala* C565 was applied into both steeping waters. These results need to be confirmed in pilot- or production scale, where wort separation can be more accurately evaluated. Wort filtration rate is influenced by several different factors such as complexes formed between proteins and pentosans, β-glucans, residual starch, and lipids (41). *P. anomala* C565 addition into the both steeping waters seemed to restrict the production of microbial β-glucanase and xylanases during malting, which might partly explain the reduced filtration rate. The microbial community, especially filamentous fungi such as fusaria, have a great influence on the malt enzyme potential and may therefore also affect wort filtration performance (27, 52, 60, 73). Furthermore, extracellular polysaccharides (EPS) produced by malt-derived bacteria and yeasts may also affect filtration perfomance (23, 31). EPS production has been reported to occur among the

yeast genera *Aureobasidium, Bullera, Cryptocccus, Pichia, Rhodotorula, Sporobolomyces, Tremella* and *Trichosporon* (9). Dense film formation (cream-colored film of biomass) due to intensive *Pichia* growth has been observed in the wine and beverage industry (62). Furthermore, Kreisz *et al*. (31) reported that malt-derived yeast polysaccharides such as mannan and glycogen may have a significant impact on the haze level of filtered beer. Therefore, precautions must be taken when selecting biocontrol agents for malting. However, $>10^6$ cfu/g *P. anomala* has frequently been observed in the normal industrial malting ecosystem without any negative consequences (35).

The possible negative impacts of *P. anomala* on filtration performance may limit its use in malting applications alone. This study suggested that the wort filtration performance could be recovered by combining *L. plantarum* E76 treatment with *P. anomala* C565. To our knowledge this is the first report in which *P. anomala* cultures were combined with *L. plantarum*. Our previous studies have shown that addition of *L. plantarum* E76 into the steeping notably improved lautering performance (24, 34). The present study also confirmed our previous findings that *L. plantarum* E76 addition enhanced xylanase and microbial β-glucanase activities. Furthermore, *L. plantarum* E76 notably restricted the growth of aerobic bacteria, especially pseudomonads known to have a negative impact on wort filtration performance (23, 33, 36). The combination of two different microbial cultures offers a possibility to use their different properties, thus making the system more robust. However, the transfer of knowledge obtained from laboratory experiments into real complex malting processes is a challenging area which definitely needs further studies. Furthermore, experiments are needed with a wider subset of barley samples.

In conclusion, this study clearly showed that yeasts naturally occurring in industrial maltings are capable of suppressing *Fusarium* growth and inhibiting the production of fungal hydrophobins inducing gushing. The combination of several treatments could result in a successful strategy for microflora management in complex cereal ecosystems such as malting. Well-characterized, malt-derived microbes can also be utilized as natural food-grade biocontrol agents in other cereal applications.

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Microbes in the tailoring of barley malt properties

Abstract
Microbes have a decisive role in the barley-malt-beer chain. A major goal of this thesis was to study the relationships between microbial communities and germinating grains during malting. Furthermore, the study provided a basis for tailoring of malt properties with natural, malt-derived microbes.

The malting ecosystem is a dynamic process, exhibiting continous change. The first hours of steeping and kilning were the most important steps in the process with regard to microbiological quality. The microbial communities consisting of various types of bacteria, yeasts and filamentous fungi formed complex biofilms in barley tissues and were well-protected. Inhibition of one microbial population within the complex ecosystem led to an increase of non-suppressed populations, which must be taken into account because a shift in microbial community dynamics may be undesirable. Both bacterial and fungal communities should be monitored simultaneously. Using different molecular approaches we showed that the diversity of microbes in the malting ecosystem was greater than expected. Even some new microbial groups were found in the malting ecosystem.

Suppression of Gram-negative bacteria during steeping was advanategous for grain germination and malt brewhouse performance. Fungal communities including both filamentous fungi and yeasts significantly contributed to the production of microbial β-glucanases and xylanases, and were also involved in proteolysis. Well-characterized lactic acid bacteria (*Lactobacillus plantarum* VTT E-78076 and *Pediococcus pentosaceus* VTT E-90390) proved to be an effective way of balancing the microbial communities in malting. Furthermore, they had positive effects on malt characteristics and notably improved wort separation.

Previously the significance of yeasts in the malting ecosystem has been largely underestimated. This study showed that yeast community was an important part of the industrial malting ecosystem. Yeasts produced extracellular hydrolytic enzymes with a potentially positive contribution to malt processability. Furthermore, several yeasts showed strong antagonistic activity against field and storage moulds. Addition of a selected yeast culture (*Pichia anomala* VTT C-04565) into steeping restricted *Fusarium* growth and hydrophobin production and thus prevented beer gushing. Addition of *P. anomala* C565 into steeping water tended to retard wort filtration, but the filtration was improved when the yeast culture was combined with *L. plantarum* E76. The combination of different microbial cultures offers a possibility to use ther different properties, thus making the system more robust. Improved understanding of complex microbial communities and their role in malting enables a more controlled process management and the production of high quality malt with tailored properties.

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Tekijä(t)
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Mikrobit ohramaltaan ominaisuuksien muokkaajana

Tiivistelmä

Mallastusprosessi voidaan määritellä ekosysteemiksi, joka koostuu itävästä jyvästä ja jyvän kanssa läheisesti elävästä monimuotoisesta mikrobiyhteisöstä. Mallastuksen mikrobiyhteisön monitorointi, ohjaus ja hallinta ovat ensiarvoisen tärkeässä asemassa, koska mikrobit vaikuttavat oleellisesti maltaan prosessiteknisiin ominaisuuksiin, mikrobiologiseen turvallisuuteen sekä lopputuotteen laatuun. Tässä tutkimuksessa perehdyttiin bakteerien, hiivojen ja homeiden vaikutuksiin ohran itämisen ja maltaan laadun kannalta. Bakteeri- ja sieniyhteisön, erityisesti hiivojen, tunnistamisessa hyödynnettiin perinteisten mikrobiologisten määritysmenetelmien lisäksi uusia molekyylibiologisia tunnistusmenetelmiä. Keskeinen tutkimuskohde oli mallastusprosessiin soveltuvien mikrobiyhteisön hallintakeinojen kartoittaminen. Tutkittiin erityisesti mallastuksen luontaisten maitohappobakteerien ja hiivojen hyötykäyttöä ekosysteemin ohjauksessa.

Tutkimus osoitti, että mikrobeilla oli keskeinen rooli mallastuksessa. Mallastusprosessissa vallitsi mikrobien kasvun kannalta edulliset olosuhteet, ja ohramatriisissa esiintyvä monimuotoinen mikrobiyhteisö pystyi mukautumaan erittäin nopeasti vaihtuviin ympäristöolosuhteisiin. Ohran liotuksen ja maltaan kuivauksen alkutunnit olivat mikrobiologisesti kriittiset pisteet. Mikrobiyhteisö osoittautui huomattavasti monipuolisemmaksi kuin aiemmin oli osoitettu. Mallastuksesta tunnistettiin uusia bakteeri- ja hiivalajeja. Mikrobiyhteisöä muokkaamalla voitiin parantaa ohran itämistä ja maltaan prosessiteknisiä ominaisuuksia. *Lactobacillus plantarum* VTT E-78076- ja *Pediococcus pentosaceus* VTT E-90390 -maitohappobakteerien lisäys ohran liotusveteen rajoitti maltaan prosessointia haittaavien bakteerien ja homeiden kasvua.

Tuotantomallastusten hiivayhteisön perusteellinen kartoitus osoitti, että tästä ryhmästä löytyi runsaasti hyödyllistä entsyymipotentiaalia. Lisäksi tiettyjen hiivojen avulla estettiin haittahomeiden, erityisesti *Fusarium*-sienten, kasvua. Mallastuksen luontaiseen mikrobiyhteisöön kuuluvan *Pichia anomala* VTT C-04565 -hiivan lisäys ohran liotusveteen esti *Fusarium*-sienten tuottamien oluen ylikuohuntatekijöiden muodostumisen mallastuksessa. Toisaalta tämä hiiva yksinään lisättynä hidasti vierteen erotusta. Epäedulliset vaikutukset maltaan prosessointiin voitiin kuitenkin poistaa, kun *P. anomala* C565 -hiivaa käytettiin yhdessä *L. plantarum* E76 -maitohappobakteerin kanssa.

Uusien monitorointi- ja ohjauskeinojen avulla on mahdollista päästä nykyistä paremmin hallittuun ja ennakoivaan prosessiin, jossa voidaan täsmällisemmin räätälöidä mallastettujen viljojen laatuparametrejä lopputuotteiden käyttäjien tarpeiden mukaisesti.

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Bacteria, yeasts and filamentous fungi are an integral part of the malting ecosystem. It is evident that microbes greatly influence the malting and brewing performance as well as the quality of malt and beer. This study surveyed the impacts of bacterial and fungal communities on barley germination and on malt properties. Furthermore, the study aimed to create possibilities for tailoring of malt quality with malt-derived lactic acid bacteria and yeasts. By modifying the microbial populations during malting, the brewing efficiency of malt could be notably improved. Well-characterized lactic acid bacteria and yeasts provided a natural way for achieving safe and balanced microbial communities in the malting ecosystem. An improved understanding of the complex microbial ecosystem and its role in malting enables a more controlled process management and the production of new malted ingredients with tailored properties.

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