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ORIGINAL PAPER

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.

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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 [53]. It is well known that barley-derived fungi and their metabolites greatly influence malt and beer quality [17, 42, 65]. *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, 61]. Contamination of the barley crop by fusaria is of concern particularly in years when poor weather conditions favor 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, 57]. 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, 41, 52]. Gushing factors are assumed to be surface-active molecules, which stabilize CO_2 bubbles in beer by forming a layer around microbubbles [46].

Our recent studies indicated that fungal proteins called hydrophobins act as the gushing factors of beer [26, 30, 52]. Hydrophobins are small, secreted, cysteine-rich proteins (100 ± 25 amino acids) that are produced by filamentous fungi [68]. Hydrophobins are among the most

abundantly produced proteins of fungi and they have various biological roles and unique properties [36]. 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 [68]. These protein films are commonly found on surfaces of aerial structures such as hyphae, conidia, and fruiting bodies [62]. A hydrophobic coating has also been proposed to have a protecting role against both desiccation 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 [67].

Strict control of incoming barley lots is vitally important in order to reject contaminated material prior to purchasing. However, malting conditions favor the growth of *Fusarium* fungi, including species which might produce mycotoxins and gushing factors during the process [22, 41, 51, 56, 57]. 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 toward 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 [37, 44]. 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 many 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, 47]. However,it is rather little known about the antifungal potential of the diverse yeast community occurring in the industrial malting ecosystem. Boivin and Malanda [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 [66]. Yeast and lactic acid bacteria often occurring 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, 70]. In addition, 4 basidiomycetous yeasts (*Cr. albidosimilis*, *Cr. curvatus*, *Cr. magnus*, and *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

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*	*Candida sake*	*C. saitoana*	*Geotrichum* sp.	*Pichia anomala*	*P. anomala*	*P. guilliermondii*	*Cryptococcus albidus*	*Cr. albidosimilis*	*Cr. curvatus*	*Cr. magnus*	*Rhodotorula pinicola*
		D-04**1014**	C-95**520**	C-04**524**	D-04**559**	C-04**564**	C-04**565**	C-04**568**	C-92**012**	C-04**508**	C-04**536**	C-04**540**	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

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. [38]. 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 micromalting equipment with a separate drum for each sample (Hulo Engineering, Helsinki, Finland). Due to 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 *P. 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 5,300*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 10^6 cfu/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 *L. plantarum* VTT E-78076 (E76) in duplicate samples. *L. plantarum* E76 strain was grown in MRS-broth (Oxoid, Basingstoke, Hampshire, UK) 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 72 h. *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) and *Pseudomonas* spp. on C–F–C agar (Oxoid Ltd.). 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 days. 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 days. Fungi were identified under a stereomicroscope on the basis of typical colony form and color. Identification was confirmed by conidia morphology with a light microscope (magnification 400×). The results are expressed as percent of kernels contaminated with fungi.

Determination of fungal hydrophobins and gushing potential

The hydrophobin levels in the malt samples were determined with competitive ELISA (Enzyme Linked Immuno-Sorben Assay) as described by Sarlin et al. [52]. Ground sample (5 g) 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, NY, USA) coated with hydrophobin extract of *F. poae* D182 Goat anti-rabbit IgG (H + L)-alkaline phosphatase (AP) conjugate (Bio-Rad Laboratories, Hercules, CA, USA) was used as a secondary antibody. p-Nitrophenyl phosphate tablets (Sigma) 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. [64]. The malt extracts were added to commercial, bottled beer (0.33 l) and pasteurized bottles were agitated for 3 days with a horizontally rotating shaker at 50 rpm [21]. The gushing positive and negative malt samples obtained from Carlsberg Research Laboratory, Copenhagen Valby, Denmark were included in the studies. After shaking, the bottles were kept still for 10 min, inverted three times and opened after 30 s. 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. [59]. 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 min at room temperature with continuous stirring (200 rpm). The flour was separated by centrifugation (1,000*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. 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 seven ascomycetous and five 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 among 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.

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

Pichia 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 (3×10^8 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.

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 ~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.

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.

The gushing test confirmed that beer gushing was prevented when *P. anomala* C565 was added into the steeping

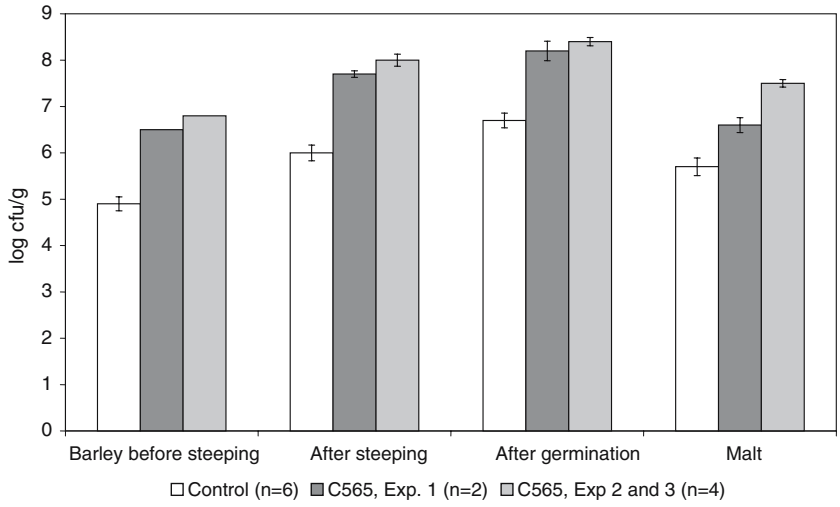

Fig. 1 Growth of indigenous yeasts (control) and inoculated *P. anomala* yeasts (C565) during the malting experiments. Duplicate grain samples were determined in each experiment. The control counts are mean values obtained from three individual malting experiments (±SD). *P. anomala* C565 counts are 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)

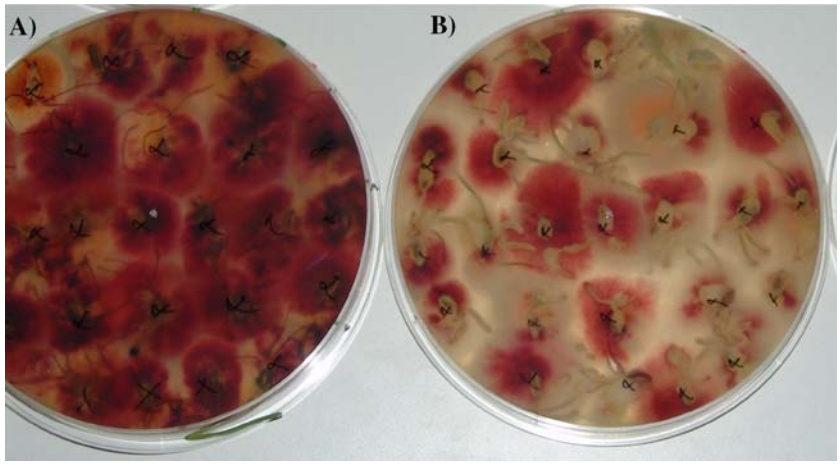

Fig. 2 *Fusarium* growth restriction by *P. anomala* C565 added to the steeping water. Kernels contaminated with *Fusarium* fungi after 2 days of malting (after steeping) on CZID plates. **a** control sample, **b** *P. anomala* C565 added to both steeping waters

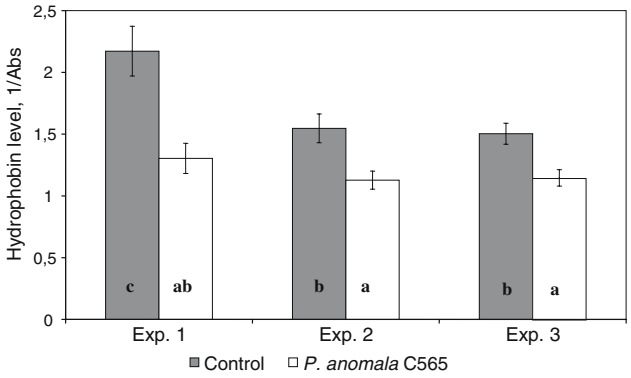

Fig. 3 Effects of *P. anomala* C565 on malt hydrophobin levels. *P. anomala* C565 was added into the first steeping water in Exp. 1 and to both the first and second steeping water in Exp. 2 and 3. Values are means of four replicates (±SD). Bars labeled with different *letters* are statistically different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

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).

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.

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 it increased considerably during malting in both control and *P. anomala* C565 samples. The final malt contained 10^7 LAB/g.

Table 2 Effects of *P. anomala* C565 on malt gushing potential

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

P. anomala C565 was added into the first steeping water in Exp. 1 and to both first and second steeping water in Exp. 2 and 3

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

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

Pichia 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

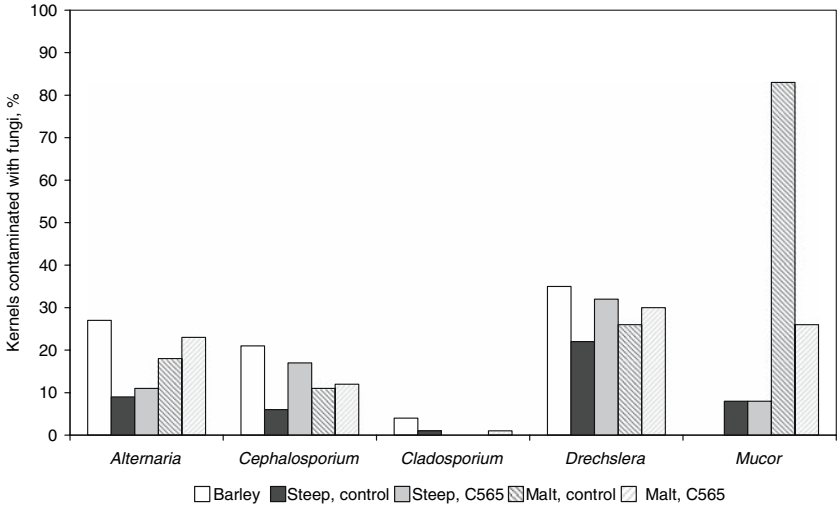

Fig. 4 Effects of *Pichia anomala* C565 added to the first and second steeping water on the occurrence of *Alternaria, Cephalosporium, Cladosporium, Drechslera*, and *Mucor* fungi in barley samples after steeping and in final malt. The values are means of the two malting experiments (Exp 2. and 3.)

Table 3 Effects of *P. anomala* C565 addition on grain germination

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

The values are mean ± standard deviation of three individual malting experiments (Exp 1–3)

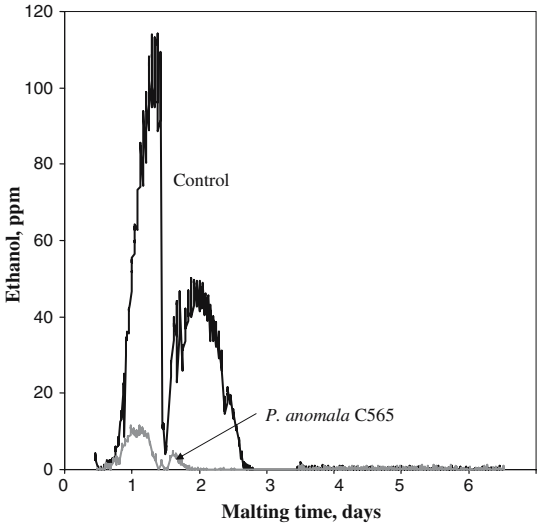

Fig. 5 Effects of *P. anomala* C565 on ethanol production during the first days of malting. The results represent one of the duplicate malting experiments (Exp. 2)

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 2 days of malting.

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.

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.

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

Table 4 Effects of *P. anomala* C565 on malt and wort (High gravity) properties

	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
Color, EBC	5.7 ± 0.2	5.5	5.6
Free amino nitrogen, mg/l	367 ± 9	404	366
Soluble nitrogen, mg/l	1,819 ± 14	1,891	1,807
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

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

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

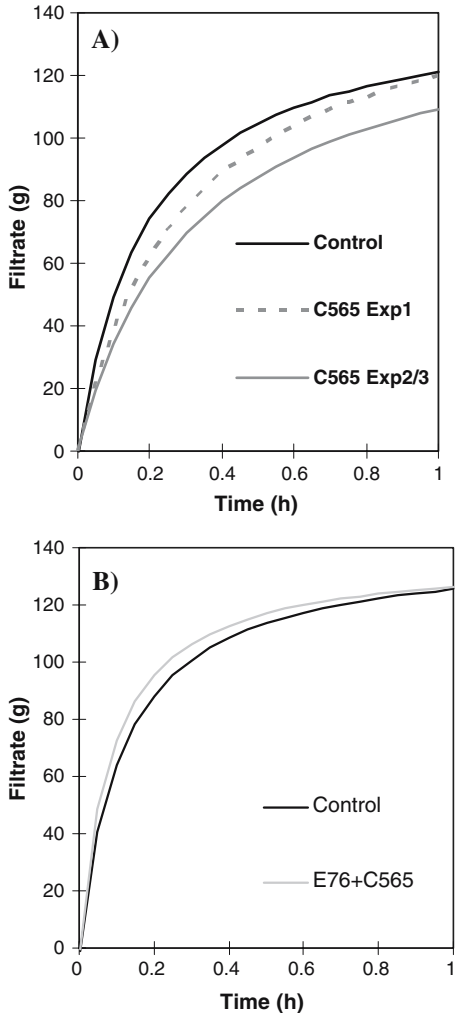

Fig. 6 Effects of *P. anomala* C565 (**a**) and of a combination of *P. anomala* C565 with *L. plantarum* E76 (**b**) on mash filterability measured as the Büchner filtration test. Values are means of triplicate (Control in **a**), duplicate (C565 Exp2/3 in **a**; E76 + C565 B) or single (C565 Exp1 in **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

bacteria, yeasts and filamentous fungi [35, 42, 49]. 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, 37, 51, 58, 63, 65]. The most negative consequences linked to intensive mould growth, especially fusaria, are the production of mycotoxins and gushing factors [52, 56].

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, 45, 50, 54, 70]. *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.

Pichia anomala is a robust organism, which is occurring naturally in plant materials such as in cereals [44]. It is traditionally used in fermented products in Africa and Asia [43]. This species is classified as safe (biosafety level 1), and is potentially a suitable biocontrol agent in a variable environment [12, 44]. *P. anomala* has previously shown antimicrobial activity against a wide range of unrelated microbes such as bacteria, yeasts, and filamentous fungi [44]. *P. anomala* J121 has been extensively studied in the preservation of moist grains (wheat, barley and oats) for animal feed [12, 18, 19, 47, 48].

We demonstrated that *P. 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 [42]. Therefore, *P. anomala* C565 was

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 × 1,000	0.203	0.278
High gravity wort analyses		
Wort extract content, w-%	16.6	16.6
Color, EBC	6.0	7.0
Free amino nitrogen, mg/l	356	392
Soluble nitrogen, mg/l	1,809	1,882
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

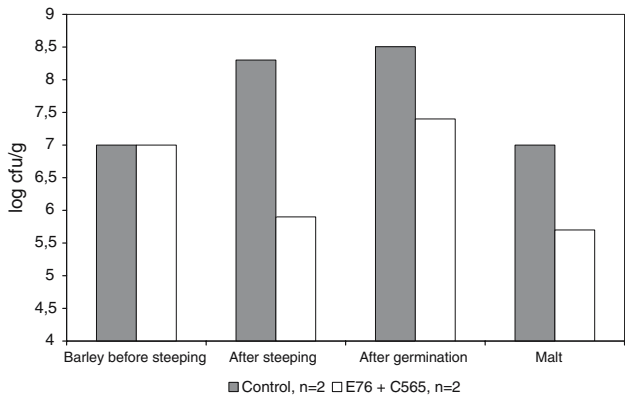

Fig. 7 Effects of *L. plantarum* E76 and *P. anomala* C565 combination on the growth of *Pseudomonas* spp. during malting. Values are means of duplicate malting samples

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 [55]. 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 among *Fusarium* species and even between strains. However, *Fusarium* diversity after *Pichia* treatment was not analyzed 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* [72]. Our further studies will be directed toward 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 [52]. 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. [52] 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 [41, 51]. 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 and 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 [41]. 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 they 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, 39, 44]. 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, it was noticed that *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, 60]. We recently reported that the ethanol detected during the first days of malting was mainly produced by the barley embryo and the aleurone cells [69]. Fermentative metabolism and concomitant ethanol production are 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, 47, 48], and expanded the list of potential application areas. However, there seemed to be a trend toward 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 [40]. *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, 51, 58, 71]. Furthermore, extracellular polysaccharides (EPS) produced by malt-derived bacteria and yeasts may also affect filtration performance [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 [60]. 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]. 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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References

1. Abildgren M, Lund F, Thrane U, Elmholt S (1987) Czapek-Dox agar containing iprodione and dichloran as a selective medium for the isolation of *Fusarium* species. Lett Appl Microbiol 5:83–86
2. Amaha M, Kitabatake K (1981) Gushing in beer. In: Pollock JRA (ed) Brewing science, vol 2. Academic, London, UK, pp 457–489
3. Blakeman J, Fokkema N (1982) Potential for biocontrol of plant diseases on the phylloplane. Annu Rev Phytopathol 20:167–192
4. Boekhout T, Robert V (eds) (2003) Yeasts in food. Beneficial and detrimental aspects. Behr's Verlag GmbH, Hamburg, Germany, p 488
5. Boivin P, Malanda M (1996) Inoculation by *Geotrichum candidum* during malting of cereals and other plants. Demande internationale publiée en vertu du Traité de Cooperation en maltière de Brevers. International Patent Cooperation Treaty (PCT) WO96/34085
6. Boivin P, Malanda M (1997) Improvement of malt quality and safety by adding starter culture during the malting process. Tech Q Master Brew Assoc Am 34:96–101
7. Chelkowski J (1991) Fungal pathogens influencing cereal seed quality at harvest. In: Chelkowski J (ed) Cereal grain: mycotoxins, fungi and quality in drying and storage. Elsevier Science Publishers, Amsterdam, The Netherlands, pp 53–66
8. Coppens T, Delcour J, Iserentant D (1996) Process for the preparation of malted cereals. International Patent Cooperation Treaty (PCT) WO9803627
9. De Baets S, Du Laing S, Vandamme E (2002) Yeast extracellular polysaccharides. In: De Baets S, Vandamme E, Steinbüchel A (eds) Biopolymers, vol 6. Polysaccharides II, Polysaccharides from eukaryotes, Wiley-VCH Verlag, Weinheim, Germany, pp 93–116
10. Doran P, Briggs D (1993) Microbes and grain germination. J Inst Brew 99:165–170
11. Douglas P, Flannigan B (1988) Microbiological evaluation of barley malt production. J Inst Brew 94:85–88
12. Druvefors U, Jonsson N, Boysen M, Schnürer J (2002) Efficacy of the biocontrol yeast *Pichia anomala* during long-term storage of moist feed grain under different oxygen and carbon dioxide regimes. FEMS Yeast Res 2:389–394
13. Ebbole D (1997) Hydrophobins and fungal infection of plants and animals. Trends Microbiol 5:405–408
14. European Brewery Convention, Analytica EBC (1998) Verlag Hans Carl Getränke-Fachverlag. Nürnberg, Germany
15. European Brewing Convention, EBC Analytica Microbiologica II (2001) Verlag Hans Carl Getränke-Fachverlag, Nürnberg, Germany (CD-ROM)
16. Filonow A, Vishniac H, Anderson J, Janisiewicz W (1996) Biological control of *Botrytis cinerea* in apple by yeasts from various habitats and their putative mechanism of action. Biol Control 7:212–220
17. Flannigan B (2003) The microbiota of barley and malt. In: Priest FG, Campell I (eds) Brewing microbiology, 3rd edn. Chapman & Hall, Kluwer Academic/Plenum Publishers, New York, USA, pp 113–180
18. Fredlund E, Druvefors U, Boysen M, Lingsten K-J, Schnürer J (2002) Physiological characteristics of the biocontrol yeast *Pichia anomala* J121. FEMS Yeast Res 2:395–402
19. Fredlund E, Ädel Druvefors U, Nilsson Olstorpe M, Passoth V, Schnürer J (2004) Influence of ethyl acetate production and ploidy on the anti-mould activity of *Pichia anomala*. FEMS Microbiol Lett 238:133–137
20. Gjertsen P (1967) Gushing in beer: its nature, cause and prevention. Brew Dig 42:80–84
21. Haikara A (1980) Gushing induced by fungi. European brewery convention monograph VI, Helsinki. Brauwelt-Verlag, Nürnberg, Germany, pp 251–259
22. Haikara A (1983) Malt and beer from barley artificially contaminated with *Fusarium* the field. In: Proceedings of the European Brewery Convention Congress, IRL Press, Oxford, UK, pp 401–408
23. Haikara A, Home S (1991) Mash filtration difficulties caused by the split barley kernels: a microbiological problem. In: Proceedings of the European Brewery Convention Congress, IRL Press, Oxford, UK, pp 537–544
24. Haikara A, Laitila A (2001) The use of lactic acid bacteria cultures in malting improvement. In: Andrews M, Andrews ME, Humphry D (eds) Plant microbial interactions: positive interactions in relation to crop production and utilisation. The Association of Applied Biologist, Warwick, UK, pp 229–238
25. Haikara A, Mattila-Sandholm T (1994) Procedure for treatment of seed material to be germinated. International Patent Cooperation Treaty (PCT) WO 9416053
26. Haikara A, Sarlin T, Nakari-Setalä T, Penttilä M (2000). Method for determining a gushing factor for a beverage. PCT/FI99/00305
27. Hoy J, MacCauley B, Fincher G (1981) Cellulases of plant and microbial origin in germinating barley. J Inst Brew 87:77–80
28. Janisiewicz W, Korsten L (2002) Biological control of postharvest diseases of fruits. Annu Rev Phytopathol 40:411–441
29. Kershaw M, Talbot N (1997) Hydrophobins and repellents: proteins with fundamental roles in fungal morphogenesis. Fungal Gen Biol 23:18–33
30. Kleemola T, Nakari-Setälä T, Linder M, Penttilä M, Kotaviita E, Olkku J, Haikara A (2001) Characterisation and detection of gushing factors produced by fungi. In: Proceedings of the European Brewery Convention Congress, Budapest. [CD-ROM] ISBN 90-70143-21-6, Fachverlag Hans Carl, Nürnberg, Germany, pp 129–138
31. Kreisz S, Wagner F, Back W (2001) The influence of polysaccharides from yeast and bacteria on the filterability of wort and beer. In: Proceedings of the European Brewery Convention Congress, Budapest. [CD-ROM] Fachverlag Hans Carl, Nürnberg, Germany, pp 1–9
32. Kurtzman C, Fell J (1998) The Yeasts, a taxonomic study. Elsevier Science B.V, Amsterdam, The Netherlands
33. Laitila A, Schmedding D, van Gestel M, Vlegels P, Haikara A (1999) Lactic acid starter cultures in malting- an application for prevention of wort filtration problems caused by bacteria present

in barley containing split kernels. In: Proceedings of the European Brewery Convention Congress, IRL Press, Oxford, UK, pp 559–566
34. Laitila A, Sweins H, Vilpola A, Kotaviita E, Olkku J, Home S, Haikara A (2006) *Lactobacillus plantarum* and *Pediococcus pentosaceus* starter cultures as a tool for microflora management in malting and for enhancement of malt processability. J Agric Food Chem 54:3840–3851
35. Laitila A, Wilhelmson A, Kotaviita E, Olkku J, Home S, Juvonen R (2006) Yeasts in an industrial malting ecosystem. J Ind Microbiol Biotechnol 33:953–966
36. Linder M, Szilvay G, Nakari-Setälä T, Penttilä M (2005) Hydrophobins: the protein-amphiphiles of filamentous fungi. FEMS Microbiol Rev 29:877–896
37. Lowe D, Arendt E (2004) The use and effect of lactic acid bacteria in malting and brewing with their relationship to antifungal activity, mycotoxins and gushing: a review. J Inst Brew 110:163–180
38. Magnusson J, Stöm K, Roos S, Sjögren J, Schnürer J (2003) Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. FEMS Microbiol Lett 219:129–135
39. Masih E, Paul P (2002) Secretion of β-glucanase by the yeast *Pichia membranifaciens* and its possible role in the biocontrol of *Botrytis cinerea* causing grey mold disease of the grapevine. Curr Microbiol 44:391–395
40. Muts G, Pesman L (1986) The influence of raw materials and brewing process on lautertun filtration. In: Proceedings of the European Brewery Convention Monograph XI, Symposium on wort production, Verlag Hans Carl, Nürnberg, Germany, pp 25–33
41. Munar M, Sebree B (1997) Gushing-a maltster's view. J Am Soc Brew Chem 55:119–122
42. Noots I, Delcour J, Michiels C (1999) From field barley to malt: detection and specification of microbial activity for quality aspects. Crit Rev Microbiol 25:121–153
43. Nout R (2003) Traditional fermented products from Africa, Latin America and Asia. In: Boekhout T, Robert V (eds) Yeasts in food. Beneficial and detrimental aspects. Behr's Verlag, Hamburg, pp 451–473
44. Passoth V, Schnürer J (2003) Non-conventional yeasts in antifungal application. In: de Winde H (ed) Functional genetics in industrial yeasts. Springer, Berlin, Germany, pp 297–330
45. Passoth V, Fredlund E, Druvefors U, Schnürer J (2005) Biotechnology,physiology and genetics of the yeast *Pichia anomala*. FEMS Yeast Res 6:3–13
46. Pellaud J (2002) Gushing: state of the art. In: Proceedings of the Xth Jean de Cleck Chair, Leuven, Belgium, pp 1–21
47. Petersson S, Schnürer J (1998) *Pichia anomala* as a biocontrol agent of *Penicillium roqueforti* in high-moisture wheat, rye, barley and oats stored under airtight conditions. Can J Microbiol 44:471–476
48. Petersson S, Jonsson N, Schnürer J (1999) *Pichia anomala* as a biocontrol agent during storage of high-moisture feed grain under airtight conditions. Postharvest Biol Technol 15:175–184
49. Petters H, Flannigan B, Austin B (1988) Quantitative and qualitative studies of the microflora of barley malt production. J Appl Bacteriol 65:279–297
50. Saligkarias I, Gravanis F, Epton H (2002) Biological control of *Botrytis cinerea* on tomato plants by the use of epiphytic *Candida guilliermondii* strains 101 and US7 and *Candida oleophila* strain I-182: II. a study on mode of action. Biol Cont 25:151–161
51. Sarlin T, Laitila A, Pekkarinen A, Haikara A (2005) Effects of three *Fusarium* species on the quality of barley and malt. J Am Soc Brew Chem 63:43–49
52. Sarlin T, Nakari-Setälä T, Linder M, Penttilä M, Haikara A (2005) Fungal hydrophobins as predictors of the gushing activity of malt. J Inst Brew 111:105–111
53. Sauer D, Meronuck R, Christensen C (1992) Microflora. In: Sauer DB (ed) Storage of cereal grains and their products. American Association of Cereal Chemists, St Paul, MN, USA, pp 313–340
54. Schena L, Nigro F, Pentimone I, Ligorio A, Ippolito A (2003) Control of postharvest rots of sweet cherries and table grapes with endophytic isolates of *Aureobasidium pullulans*. Postharvest Biol Technol 30:209–220
55. Schmidt H (1991) Cereal grain structure and the way in which fungi colonize kernel cells. In: Chelkowski J (ed) Cereal grain: mycotoxins, fungi and quality in drying and storage. Elsevier Science Publishers, Amsterdam, The Netherlands, pp 1–22
56. Schwarz P, Casper H, Beattie S (1995) Fate and development of naturally occurring *Fusarium* mycotoxins during malting and brewing. J Am Soc Brew Chem 53:121–127
57. Schwarz P, Beattie S, Casper H (1996) Relationship between *Fusarium* infestation of barley and the gushing potential of malt. J Inst Brew 102:93–96
58. Schwarz P, Jones B, Steffenson B (2002) Enzymes associated with *Fusarium* infection of barley. J Am Soc Brew Chem 60:130–134
59. Sjöholm K, Pietilä K, Home S, Aarts R, Jaakkola N (1994) Evaluation of the lautering performance of malt. Monatsschr Brauwiss 47:165–171
60. Sponholz W-R (1993) Wine spoilage by microorganisms. In: Fleet G (ed) Wine microbiology and biotechnology. Harwood Academic Publishers, Chur, Swizerland, pp 395–420
61. Steffenson B (1998) *Fusarium* head blight of barley: epidemics, impact, and breeding for resistance. Tech Q Master Brew Assoc Am 35:177–184
62. Talbot N (1999) Fungal biology. Coming up for air and sporulation. Nature 398:295–296
63. Tuomi T, Laakso S, Rosenqvist H (1995) Plant hormones in fungi and bacteria from malting barley. J Inst Brew 101:351–357
64. Vaag P, Riis P, Knudsen A-D, Pedersen S, Meiling E (1993) A simple and rapid test for gushing tendency in brewing materials. In: Proceedings of the European Brewery Convention Congress, IRL Press, Oxford, UK, pp 155–162
65. van Nierop S, Rautenbach M, Axcell B, Cantrell I (2006) The impact of microorganisms on barley and malt quality- A review. J Am Soc Brew Chem 64:69–78
66. Vaughan A, O'Sullivan T, van Sinderen D (2005) Enhancing the microbiological stability of malt and beer—a review. J Inst Brew 111:355–371
67. Wessels J (1996) Fungal hydrophobins: proteins that function at an interface. Trends Plant Sci 1:9–15
68. Wessels J (1997) Hydrophobins: proteins that change the nature of the fungal surface. In: Poole RK (ed) Advances in microbial physiology. Academic, London, UK, pp 1–45
69. Wilhelmson A, Laitila A, Vilpola A, Olkku J, Kotaviita E, Fagerstedt K, Home S (2006) Oxygen deficiency in barley (*Hordeum vulgare*) grain during malting. J Agric Food Chem 54:409–416
70. Wisniewski M, Biles C, Droby S, McLaughlin R, Wilson C, Chalutz E (1991) Mode of action of the postharvest biocontrol yeast, *Pichia guilliermondii*. I Characterization of attachment to Botrytis cinerea. Physiol Mol Plant Pathol 39:245–258
71. Yin X, MacGrecor A, Clear A (1989) Field fungi and β-glucan solubilase in barley kernels. J Inst Brew 95:195–198
72. Yli-Mattila T, Paavanen-Huuhtala S, Parikka P, Konstantinova P, Gagkaeva T (2004) Molecular and morphological diversity of Fusarium species in Finland and north-western Russia. Eur J Plant Pathol 110:573–585