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Investigation of the microbiota associated with ungerminated and germinated Norwegian barley cultivars with focus on lactic acid bacteria

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ABSTRACT

The microbial community of ungerminated and germinated barley grains from three different cultivars grown at four different locations in Norway was investigated by culture dependent and culture independent methods. Lactic acid bacteria (LAB) was focused in this study and was isolated from germinated barley. The number of LAB ranged between 2.8 and 4.6 log cfu/g in ungerminated grains and between 4.9 and 6.3 log cfu/g in germinated grains. In total 66 out of 190 isolates were Gram+, catalase-negative and presumptive LAB. The LAB isolates were by 16S rRNA sequencing identified to be *Carnobacterium maltaromaticum* (6), *Lactococcus lactis* (2), *Enterococcus* sp. (1) and *Leuconostoc* sp. (57). Germination significantly influenced the bacterial composition. Regarding the different cultivars and growth places no significant difference in bacterial composition was seen. The most abundant bacterial genus was *Pantoea* (18.5% of the total sequences), followed by *Rhizobium* (10.1%) and *Sphingomonas* (9.9%). Fungal composition was significantly influenced by the germination process and the cultivation place, but no significant difference in fungal composition was detected between the 3 cultivars. The most abundant fungal genera were *Cryptococcus* (43.8% of all the sequences), *Cladosporium* (8.2%), *Pyrenophora* (7.4%) and *Vagicola* (6.3%). This study revealed knowledge of barley grain associated microbes of Norwegian barley that can be useful to control the malt quality. Germination affected both bacterial and fungal microbiota composition. No difference in bacterial microbiota composition was seen regarding cultivars and cultivation place, however, the fungal microbiota composition was significantly influenced by the cultivation place. Differences in fungal community of ungerminated and germinated barley samples of different geographical locations were more pronounced than differences in bacterial communities.

1. Introduction

Barley is important both as a feed crop, but also as the predominant raw material for production of malt for brewing (Noots et al., 1999). Barley grains carry a numerous, variable and complex microbial population that consists of bacteria, yeasts and moulds. The microbiota is mainly dominated by mesophilic and psychotrophic microorganisms. Production factors such as cultivation, harvest and storage of the cereal have an impact on the microbiota of the grain (Chen et al., 2016; Li et al., 2018; Noots et al., 1999). It has also been shown that the microbial community of barley grains influences the malt quality and subsequently also the wort and beer quality (Chen et al., 2016; Laitila et al., 2007).

The microorganisms of cereals during growth, harvest and storage consists of bacteria, yeasts and moulds, however, they are dominated by moulds (Adams et al., 2016). It is convenient to divide the moulds into two groups of fungi. The field fungi, being well adapted to the sometimes rapidly changing weather conditions and the storage fungi being more adapted to the more constant conditions and generally low water activities (a_w 0.68–0.8). The field fungi generally do not grow below a_w 0.9. The most important genera of storage fungi are *Penicillium* and *Aspergillus*. Different genera as *Cladosporium*, *Alternaria* and *Epicoccum* are examples of genera of field fungi. *Fusarium* may be found both on the field and during storage of cereals, especially under moist conditions. Yeasts are isolated from the barley grains, malt barley or from the process. The most commonly found yeasts in barley grains and malting ecosystems include the Ascomycota yeasts belonging to the genera *Aureobasidium*, *Candida*, *Clavispora*, *Exophiala*, *Galactomyces*, *Hanseniaspora*, *Issatchenkia*, *Pichia* and *Saccharomyces* and the Basidiomycota yeasts such as, *Bulleromyces*, *Cryptococcus*, *Filobasidium*, *Rhodotorula*,

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Sporobolomyces and *Trichosporon* (Korhola et al., 2014; Laitila et al., 2006b).

The malting process starts by moistening the barley grains by steeping in water, continues with germination and ends with kilning (Adams et al., 2016; Noots et al., 1999). The rapid hydration process and secretion of nutrients into the water leads to growth of microorganisms from the grains. Also, the malting equipment may influence the evolution of the barley microbiota during malting. A specific microbiota may occur on the malting equipment that contaminates the grains. The growth of the microorganisms during the malting process has been described in many studies (Li et al., 2018; Noots et al., 1999; O'Sullivan et al., 1999). Some microorganisms may be washed off during steeping, however, the microbial count significantly increase during the steeping process and remain high during germination. The bacterial population of barley before malting was dominated by Gram-negative coliforms (11.5%) and pseudomonads (5%), with lactic acid bacteria (LAB) constituting a small minority of the total bacterial count. The dominating Gram-negative bacteria were identified as *Serratia*, *Erwinia*, *Enterobacter*, *Pseudomonas* and *Aeromonas*. Lactic acid bacteria and *Geotrichum candidum* were predominant contaminants during steeping and germination in industrial malthouses (Noots et al., 1999). *Leuconostoc* sp. were the dominating LAB during the malting process, however, other LAB were also identified as *Enterococcus*, *Lactobacillus* and *Streptococcus* (O'Sullivan et al., 1999). LAB increased to high numbers (8 log cfu/g) during steeping and remained high during the germination period (7.8 log cfu/g). Booysen et al. (2002) isolated and identified LAB throughout malting of two barley cultivars. The strains were identified to species level and the predominant bacterial species in both cultivars before steeping were *Leuconostoc lactis*, *Leu. argentinum* and *Weisella confusa*. Kilned malt of one of the cultivars contained predominantly *Leu. argentinum*, whereas the other contained mainly *Lactococcus lactis*.

Most studies have been using conventional quantitative culture dependent methods to study the microbial communities, however, these methods are biased towards the selective growth media being used causing dominance of the fast-growing microorganisms on this specific media. Culture independent methods as high-throughput sequencing are now available and commonly used to assess the microbial community diversity and dynamics in food-based ecosystems.

The objective of the paper was to study the microbial community of ungerminated and germinated barley grains from three different cultivars grown at four different geographical parts in Norway, with focus on LAB and their isolation and identification from the germinated grains. Nothing is known about the microbiota of Norwegian barley cultivars during malting and if the localization of the farms influences the microbial community or if the different cultivars influence the microbiota. Both culture dependent and culture independent methods such as high-throughput sequencing approaches were applied in the study.

2. Materials and methods

2.1. Barley samples, steeping and germination

Barley samples from three cultivars, Fairytale (two-row), Helium (two-row), and Brage (six-row), were grown and harvested at four farms situated in different geographical parts of east Norway (Hagen, Hamar (60.81 N 11.20 E), Apelsvoll, Toten (60.70 N 10.87 E), Aas (59.67 N 10.76 E), Rød, Råde (59.35 N 10.87 E)) in the autumn of 2016.

The barley samples, 30–40 g, were steeped and germinated at 16 °C in separate chambers in malting equipment from Custom Laboratory Products (Milton Keynes, England) using intervals with steeping and aeration for 8 h and 16 h, respectively. The steeping and aeration intervals were repeated twice, before 2 h of steeping and 46–72 h of germination. Germination was terminated when the average leaf sprout was 60–80% of the grain length. The grains were turned firmly throughout the malting process. Samples were taken at the end of the germination process, before kiln drying. Malt at this stage is called green malt.

2.2. Microbiological sampling of barley

From each ungerminated and germinated barley sample, 10 g were homogenized with 90 mL Ringer's solution (Oxoid Ltd., Hampshire, England) in an autoclaved Waring blender (Waring Commercial, Stanford, USA) for 3 min (Fairytale and Brage) and 6 min (Helium). Serial dilutions were made in Ringer's solution before plating on different media for viable counts. Aerobic mesophilic bacteria were enumerated on plate count agar (PCA, Oxoid) and lactic acid bacteria on De Man Rogosa Sharpe agar (MRS, Oxoid), both incubated for 2–4 days at 30 °C. Lactobacilli were enumerated on *Lactobacillus* selective agar (LBS agar, Becton Dickinson Microbiology Systems, Sparks, MD, USA) incubated in 10% CO_2 atmosphere (Heraeus CO_2 incubator, W.C. Heraeus GmbH, Hanau, Germany) for 3–4 days at 30 °C. Yeast and moulds were enumerated on Rose Bengal Chloramphenicol agar (RBC agar, Merck KGaA, Darmstadt, Germany) incubated at 25 °C for 5 days.

2.3. Isolation of lactic acid bacteria and preliminary characterization

Lactic acid bacteria were isolated from the germinated barley samples. From LBS agar and MRS agar, 10–23 single colonies were randomly picked from each barley cultivar from the four farms before transfer to MRS broth (Oxoid). There was less growth on the LBS agar plates, however, at least 10 colonies were picked from each sample. In total, 190 isolates were sub-cultured to purity on MRS agar before freezing and storage of the cultures in MRS medium containing 15% (v/v) glycerol. The bacteria were inoculated (1%, v/v) in MRS broth before incubation overnight at 30 °C at least twice before use.

Gram reaction, catalase reaction (3% H_2O_2) and morphology of the isolates by phase contrast microscopy were conducted on pure cultures.

2.4. Culture-dependent 16S rRNA sequence analysis

Genomic DNA from 39 out of 70 isolates was extracted from cells harvested from overnight cultures. DNA was extracted using E.Z.N.A.® Plasmid DNA Mini Kit I (Omega Bio-tek, Norcross, Georgia) according to manufacturer's instructions with some modifications. Bead beating was introduced after resuspension of pellet in 400 μL Solution 1/RNase A and transfer to FastPrep-tubes containing 0.4 g acid-washed glass beads (≤106 μm, Sigma-Aldrich Co. LLC., USA). The cells were lysed in a cell disrupter (FastPrep®-24 instrument, MB Biomedicals LLC, USA) for 20 s at 6 m/s. The reaction mixture was then centrifuged at 3500 rpm for 2 min and the supernatant transferred into a new Eppendorf tube. Subsequent DNA recovery was done according to the manufacturer's instructions. The quality and concentration of DNA were determined using a nanoDrop 2000 spectrophotometer (ND-2000 UV-VIS spectrophotometer, Thermo Fisher Scientific, Wilmington, USA). Universal primers, 11 F (5′-TAACACATGCAAGTCGAACG 3′) and 5R (5′GGTT ACCTTGTTACGACTT-3′) (*Escherichia coli* positions 50–70 (Edwards et al., 1989) and 1492–1510 (Eden et al., 1991), respectively) were used for amplification of a 1460 bp DNA fragment of the 16S rRNA gene. The PCR reactions were accomplished using One *Taq*® DNA Polymerase (New England BioLabs, Hertfordshire, England, UK) according to manufacturer's procedure. The PCR reactions were carried out in a Peltier Thermal Cycler (PTC-200, MJ Research Inc., Quebec, Canada) using the following program: 1 cycle of denaturation at 94 °C for 3 min; 34 cycles consisting of denaturation at 94 °C for 15 s, primer annealing at 58 °C for 15 s and elongation at 68 °C for 1.5 min; a final extension step at 68 °C for 5 min. The PCR products were purified using E.Z.N.A. ® Cycle Pure Kit (Omega Bio-tek) according to the procedure recommended by the supplier. Automated DNA sequencing was provided by GATC Biotech (Konstanz, Germany) using the universal primers 5R (Edwards et al., 1989) and 11F (Eden et al., 1991). Sequences were edited using BioEdit software version 7.0.5.3 (Hall, 1999; Ibis Biosciences, Carlsbad, CA,

USA) and were compared in GenBank using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/blast/; Hall, 1999). A phylogenetic tree was constructed from the aligned 16S rRNA sequences using the MAFFT alignment program version 7 (https://mafft.cbrc.jp/alignment/server) (Katoh et al., 2019) and distance between sequences was calculated using the Tamura-nei method. The tree was visualized by the neighbour-joining method using Geneious software version 10.0.7.

2.5. Microbiota composition of barley

2.5.1. DNA extraction, 16S rRNA and ITS region library construction and sequencing

Bacterial and fungal microbiota from ungerminated and germinated barley grains were studied by next generation sequencing (NGS) of the 16S rRNA gene and the internal transcribed spacer (ITS) region, respectively. Genomic DNA was extracted from 10 mL of the frozen homogenized Waring blender solution made from 10 g of barley and 90 mL Ringer's solution. Liquid nitrogen was not used in the preparation of samples in microbiome studies and this might have affected the microbiota composition. The solution was centrifuged at 8000 rpm for 10 min at 4 °C and the pellet was washed three times with 1 mL of 2% (w/v) sodium citrate buffer before resuspension in 200 μL lysis buffer (20 mM Tris·HCl (pH 8.0), 2 mM sodium EDTA, 1.2% Triton® X-100). DNA was extracted using Mag midi kit (LGC Limited, Teddington, UK) according to manufacturer's instructions. Briefly, the cells were lysed with bead-beating in a FastPrep®-24 instrument using 0.5 g acid-washed glass beads (≤106 μm) with 3 repetitions consisting of shaking (50 s) and cooling (1 min) on ice. The reaction mixture was then centrifuged at 13000 rpm for 1 min and the supernatant (50 μL) transferred to a 96-well plate. Subsequent DNA extraction was done according to the manufacturer's instructions of the Mag midi kit, except using 50 μL elution buffer BLm instead of 63 μL. Sequencing libraries were constructed according to the method described by Porcellato and Skeie (2016).

Amplification of the variable regions V3 and V4 of the bacterial 16S rRNA gene was performed using the universal primer Uni340F (5′-CCTACGGGRBGCASCAG-3′) and Bac806R (5′-GGACTACYVGGGTATCTAAT-3′) (Takai and Horikoshi, 2000), while for the ITS region of fungal operon the primer set was composed of the primer BITS (5′ACCTGCGGARGGATCA–3′) and B58S3 (5′–GAGATCCRTTGYTRAAAGTT–3′) (Bokulich and Mills, 2013). The PCR reaction for amplification of DNA was performed as described by Porcellato and Skeie (2016) except for using 2 μL of DNA and annealing temperatures of 53 and 55 °C, for the bacterial and fungal library, respectively. After PCR amplification, 20 μL of PCR product was purified by means of 0.6 and 0.8 g× of Agencourt AMPure XP beads (Beckman Coulter, Inc., Brea, CA, USA) for the bacterial and fungal library, respectively. The purification was performed according to the manufacturer's instruction. The first PCR of the library preparation was done using a qPCR system to quantify the genomic DNA. Five of the purified PCR products were used as template for the second PCR using customized primers with unique sample barcodes according to Porcellato and Skeie (2016). Both the bacterial and fungal libraries were purified and normalized using SequalPrep™ Normalization plate (Thermo Fischer Scientific, Oslo, Norway). The two libraries were quantified using the Kapa library quantification kit (Kapa Biosystems, Wilmington, MA, USA) and sequenced using an Illumina Miseq platform (Illumina, San Diego, CA, USA) with a 300 bp paired-end sequencing kit (Illumina). Sequence data are available at the European Nucleotide Archive under the accession number PRJEB35861.

2.6. Bioinformatic analysis of the bacterial 16S rRNA gene and fungal ITS region libraries

Sequence data quality was evaluated using FastQC version 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Reads were quality filtered (maxEE = 2) and trimmed using the DADA2 R package (Callahan et al., 2016) using sequence truncation length of 260 and 240 bp for forward and reverse reads, respectively. No truncation was performed for the fungal sequences. The error model in DADA2 R package was created using 0.5 million random filtered reads. Sequence variants (SV) was inferred using the DADA2 R package algorithm (Callahan et al., 2015) and removal of chimeras was performed using the function "removeBimeraDenovo" in the DADA2 R package. SV with less than 100 sequences and SV shorter than 375 bp were removed from the final bacterial table, while fungal SV were removed if the length was less than 150 bp. Taxonomy was assigned using the Decipher R package (Murali et al., 2018) against the SILVA SSU database release 132 (Quast et al., 2013) and the UNITE ITS database using the confidence threshold >60%. Normalization of the SV table was performed using the metagenomeSeq R package using the cumNorm function (Paulson et al., 2013). Bacterial and fungal richness were calculated with the Chao1 estimator, while diversity was calculated with the Shannon index from the Vegan R package (Chao, 1987; Dixon, 2003). Permutational multivariate analysis of variance (function *Adonis* in the Vegan R package) (Oksanen et al., 2017) was used to evaluate if the microbial composition was influenced by the different factors (place of harvest, the germination step and the cultivar) using the Bray-Curtis dissimilarity matrix. Principal coordinate analysis was used as ordination method to visualize the beta diversity between samples using the Bray-Curtis dissimilarity matrix. Single taxa at the genera level were evaluated for significant difference between the germinated and ungerminated samples using a paired Wilcoxon signed rant test (significant different taxa were identified with Wilcoxon adjuster $p < 0.05$).

3. Results

3.1. Evolution of microbial cell counts during germination

The microbial cell numbers were determined before and after germination of the 12 barley samples, see Table 1. The countable total cell numbers of the barley samples from Rød and Apelsvoll before steeping ranged from 6.0 to 7.5 log cfu/g, however, the total cell numbers before steeping was uncountable on samples from Aas and Hagen (≥5.5 log cfu/g). The total cell numbers on the germinated grains varied between 7.8 and 8.6 log cfu/g. The increase in total cell numbers ranged from 0.7 to 2.0 log cfu/g during the germination process in the countable samples. The countable number of bacteria on MRS agar, presumptive lactic acid bacteria, in samples from Rød and Apelsvoll ranged between 2.8 and 4.6 log cfu/g on the barley grains before steeping and on the germinated grains between 4.4 and 6.3 log cfu/g. Most of the samples showed an increase in LAB number during germination ranging from 1.0 to 2.2 log cfu/g. The number of bacteria counted on LBS agar, presumptive *Lactobacillus*, was very low in all samples (≤1.0–1.3 log cfu/g) before steeping and below 2 log cfu/g in samples from Rød and Apelsvoll after germination. However, in the countable germinated samples from Aas and Hagen the cell numbers on LBS agar ranged from 2.5 to 6.3 log cfu/g. The number of countable moulds and yeasts ranged from 2.5 to 5.2 log cfu/g in the ungerminated barley grains, and from 4.3 to 7.2 log cfu/g in the germinated grains. An increase during the malting process of 0.7 to 3.5 log cfu/g was observed in the fungi cell numbers.

3.2. Phenotypic characterization and culture dependent identification by 16s rRNA sequencing

There were randomly picked 10–23 colonies from the green malt from the four places, giving in total 190 pure cultures after streaking until purity the selected colonies from the MRS- and LBS agar plates. Characterization and identification of the presumptive LAB isolates are shown in Table 2. Sixty-six of the green malt isolates were Gram-positive and catalase-negative bacteria. Based on microscopy, cultivation place and cultivar, 36 of the 66 isolates were selected for identification by 16S rRNA

Table 1
Microbial growth on three cultivars of barley grains from four different places. Counts on: PCA agar for aerobic mesophiles, MRS agar for lactic acid bacteria, LBS agar for presumptive lactobacilli, Rose Bengal agar for moulds and yeasts.

Place	Cultivar	Viable counts (log cfu/g)							
		PCA		MRS		LBS		Rose Bengal	
		Ungerm.	Germ	Ungerm.	Germ.	Ungerm.	Germ.	Ungerm.	Germ.
Aas	Fairytale	>5.48	8.30	3.60[a]	5.77	<1.00	<4.00	3.90	7.23
	Helium	>5.48	7.79	4.59[a]	5.54	<1.00	5.32	5.08	6.63
	Brage	>5.48	8.11	3.66[a]	5.83	<1.00	<2.00	5.15	5.90
Hagen	Fairytale	>5.48	8.20	>5.48[a]	6.32	<1.00	6.26	>5.48	6.93
	Helium	>5.48	8.30	3.77[a]	5.43[a]	<1.00	<4.00	3.82	7.20
	Brage	>5.48	8.18	3.34[a]	6.30	<1.00	2.48	>5.48	5.40
Rød	Fairytale	>7.48	7.93	4.63[a]	4.38[a]	<1.00	<2.00	<3.00	5.96
	Helium	7.45	8.18	3.72[a]	4.88[a]	<1.00	<2.00	4.45	6.67
	Brage	6.78	8.08	3.43[a]	4.83[a]	<1.00	<2.00	4.32	6.00
Apelsvoll	Fairytale	6.04	7.95	2.78[a]	4.38	<1.00	<2.00	3.61	4.30
	Helium	6.70	8.56	4.18[a]	6.26	<1.00	<2.00	3.65	5.72
	Brage	6.32	8.34	3.38[a]	>6.48	1.3	<2.00	2.54	6.04

[a] Domination of moulds and yeasts.

Table 2
Identification of lactic acid bacteria isolated from the three different cultivars of barley grown at four different places in Norway after germination by 16S rRNA sequencing and microscopy.

Place	Cultivar	Isolates G+/Cat-[a]		Identification
Aas	Fairytale	16	2	2 *Leuconostoc citreum/holzapfelii*
	Helium	22	22	5 *Leu. citreum/holzapfelii* 17 *Leuconostoc*[b]
	Brage	15	0	–
Hagen	Fairytale	28	28	12 *Leu. citreum/holzapfelii* 16 *Leuconostoc*[b]
	Helium	17	0	–
	Brage	12	11	1 *Leu. citreum/holzapfelii* 3 *Leuconostoc pseudomesenteroides/ mesenteroides* 1 *Lactococcus lactis* 6 *Carnobacterium maltaromaticum*
Rød	Fairytale	9	0	–
	Helium	8	0	–
	Brage	10	0	–
Apelsvoll	Fairytale	13	1	1 *Enterococcus*
	Helium	23	2	1 *Lactococcus lactis* 1 *Leuconostoc*[b]
	Brage	17	0	–

[a] Number of Gram-positive and catalase-negative isolates.
[b] Presumptive genus based on microscopy.

sequencing. Microscopy showed a high number of coccoid-rod shaped bacteria (57), 6 rod shaped bacteria and 3 cocci shaped bacteria isolates. All rod shaped and cocci shaped bacteria were identified by 16S rRNA sequencing to be *Carnobacterium maltaromaticum* (6), *Lactococcus lactis* (2) and *Enterococcus* (1). A representative selection of 23 coccoid-rod shaped bacteria were all identified by sequencing to belong to the *Leuconostoc* genus, and to the species *Leuconostoc citreum/holzapfelii* (20) and *Leuconostoc pseudomesenteroides/mesenteroides* (3), respectively. No presumptive LAB were isolated from Rød from any of the cultivars and none from Brage grown at Aas, Helium grown at Hagen and Brage grown at Apelsvoll. In general, very few LAB were found at Apelsvoll.

3.3. Microbiota composition of barley

To evaluate the microbial composition in barley before and after germination, 16S rRNA and ITS sequencing were applied. A total of 206,627 and 1,265,513 good quality sequences were obtained from the 16S rRNA and ITS libraries, respectively. The sequences were clustered in 404 bacterial OTUs and 398 fungal OTUs.

The analysis of the alpha diversity indexes indicated that the germination step influences the bacterial composition, but not the fungal composition. Germinated barley samples showed a higher number of estimated bacterial OTUs and a higher bacterial diversity compared to ungerminated samples (Fig. 1A; Supplementary Table S1). On the contrary the estimated number of fungal OTUs was higher for ungerminated samples while no difference between the germinated and ungerminated fungal diversity was detected (Fig. 1B, Supplementary Table S1).

Taxonomical classification demonstrated the presence of seven bacterial phyla, namely Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Patescibacteria, Cyanobacteria and Acidobacteria in the germinated and ungerminated barley samples (Fig. 2A). Small differences according to cultivars and cultivation place of the samples at the phylum level were observed. Proteobacteria was the dominating phylum in both germinated and ungerminated samples with relative abundance in the range 58–90% and 48–80%, respectively.

Bacterial composition of the samples at the genus level was significantly influenced by the germination step (Adonis $p < 0.05$), while no significant differences in bacterial composition were detected between the cultivar and the place or between these 2 factors and interaction with the germination step tested in a model comparing germinated and ungerminated samples (Fig. 2B; Supplementary Fig. S1). The most abundant bacterial genus was *Pantoea* (18.5% of the total sequences), followed by *Rhizobium* (10.1%) and *Sphingomonas* (9.9%). Both *Pantoea* and *Rhizobium* abundances were significantly different between the germinated and ungerminated samples. *Rhizobium* was more abundant in the germinated samples while *Pantoea* had a higher abundance in the ungerminated samples compared to the germinated (Fig. 4). Other genera obtained a significant different abundance between the germinated and the ungerminated samples. *Pseudomonas* and *Paenibacillus* were more abundant in samples before germination while *Chryseobacterium*, unclassified *Microbacteriaceae*, unclassified *Rhizobiaceae*, *Acinetobacter*, unclassified *Burkholderiaceae*, *Brevundimonas*, *Methylobacterium*, *Microbacterium*, and *Flavobacterium* were more abundant after germination.

At the phylum level, Basidiomycota and Ascomycota were the main fungal phyla in both germinated and ungerminated samples (Fig. 3A). In the germinated samples, both Basidiomycota (5–85%) and Ascomycota (15–95%) phyla were observed and some differences were observed related to cultivars and place. Basidiomycota dominated in the ungerminated samples (59–95%) and there was little difference related to cultivars and cultivation place at the phylum level.

Fungal composition of the germinated and ungerminated grains samples at the genus level was significantly influenced by the germination process and the place (Adonis $p < 0.05$) where the grain was

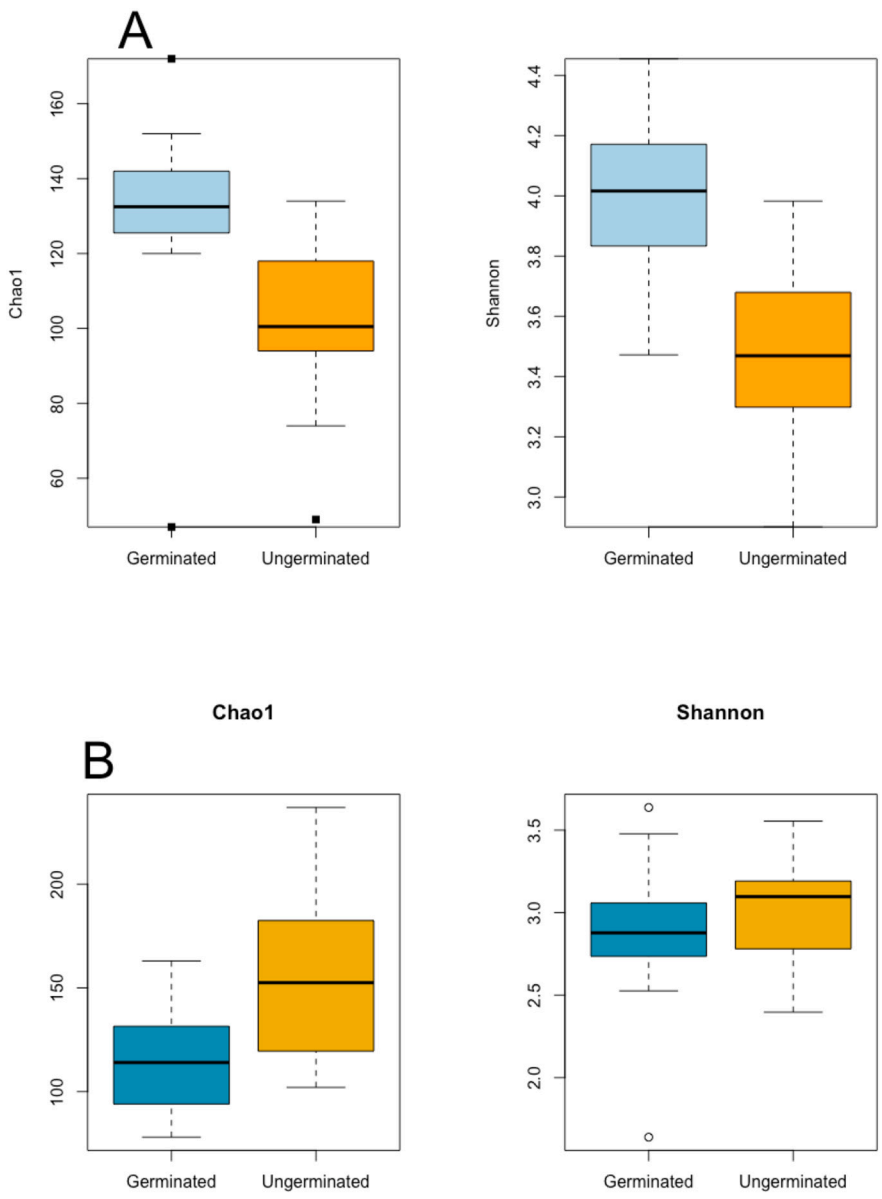

Fig. 1. Alpha diversity analysis calculated of the dataset, measured as Chao1 and Shannon diversity indexes. (A) Bacterial OTUs based on 16S rRNA sequences. (B) Fungal OTUs based on ITS-sequences.

harvested (Fig. 3B; Supplementary Fig. S1). No significant differences in fungal composition were detected between the cultivars (Adonis $p >$ 0.05). However, difference in relative abundance between the grain cultivars was detected for example in germinated samples from Aas, Apelsvoll and Rød and in ungerminated samples from Aas, Hagen and Rød. The most abundant fungi genera were *Cryptococcus* (43.8% of all the sequences), *Cladosporium* (8.2%), *Pyrenophora* (7.4%) and *Vagicola* (6.3%). No significant differences were found in relative abundance between the germinated and ungerminated grain samples for the dominant genera *Cryptococcus*, while the germination process significantly reduced the relative abundance of the genus *Cladosporium* (Fig. 5). The relative abundance of 3 other genera (*Sporobolomyces*, *Naganishia* and *Phaeosphaeria*) was also influenced by the germination process where *Sporobolomyces* and *Phaeosphaeria* were more abundant in the ungerminated samples and *Naganishia* in the germinated samples (Fig. 5).

4. Discussion

Barley for malt is grown in a diverse range of environments and geographic locations all over the world. Barley provides an ecological niche for a diverse range of microorganisms, but the microbiota of different barleys is remarkable similar to each other, and to other cereals (Kaur et al., 2015). This work was undertaken to investigate the microbiota of ungerminated and germinated barley grains cultivated at different geographical parts of east Norway. The microbial number of total aerobic bacteria, presumptive lactic acid bacteria, and moulds and yeasts were determined on the grains before germination and after germination. In this study two two-row cultivars, Fairytale and Helium, and one six-row cultivar, Brage, were studied. The aerobic bacterial counts were higher than the fungal cell numbers before and after germination. There were also large variations in the number of fungi in both ungerminated and germinated samples, regarding both place of growth but also regarding the different cultivars, however, all samples showed the highest number of fungi in the germinated samples. The number of presumptive LAB were much lower in the ungerminated samples (2.8–4.6 log cfu/g) compared to the germinated samples (4.4–6.3 cfu/g), and this observation was even clearer regarding the number of presumptive lactobacilli with <1–1.3 log cfu/g in the ungerminated samples and <2–6.26 log cfu/g in the germinated ones. In general, a lower LAB number was seen on germinated samples from Rød

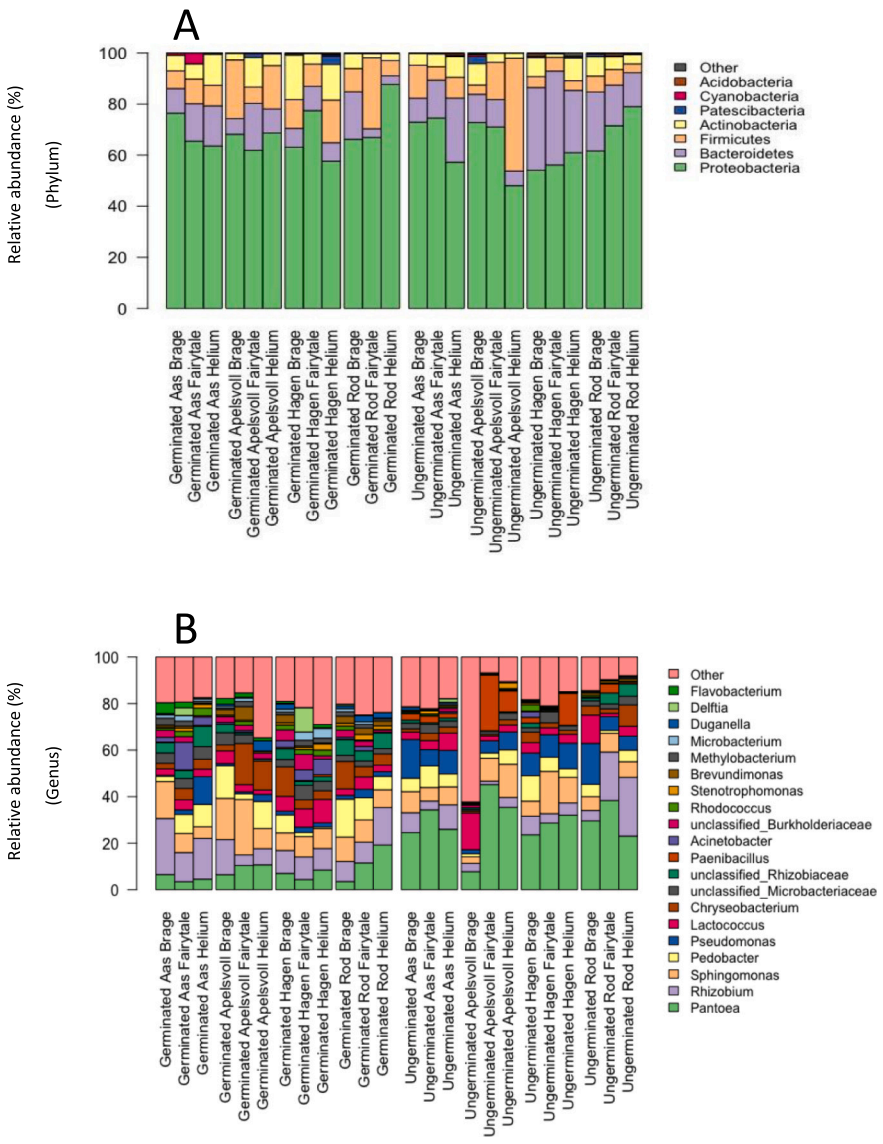

Fig. 2. Relative abundance of (A) bacterial phyla and (B) the 20 most representative bacterial genera in the samples of germinated and ungerminated barley cultivars cultivated in four geographical regions in Norway. Other: indicate phyla and genera with relative abundance <0.05%.

compared with the other growth places. Li et al. (2018) studied the microbial community dynamics of Dan'er barley grain during an industrial malting process by using culture dependent and culture-independent methods. Dan'er is a two-row barley cultivar and is one of the most used barley cultivars in China. The aerobic bacterial counts were much higher than the fungal cell numbers throughout the whole malting process, as observed in this study. The aerobic bacterial count peaked at the end of the germination with 7.9 log cfu/g. The yeast counts also peaked at this stage with 7.0 log cfu/g, however, the filamentous fungi showed in general numbers that were much lower during the malting process and about the same numbers were seen in the green malt (3.8 log cfu/g) as in the dried malt (4 log cfu/g). Some mould growth was seen on the MRS plates of the Norwegian barley samples. The mould growth was lower on the germinated samples than in the ungerminated ones.

In this study the focus was on LAB so only isolates from MRS- and LBS-agar were studied in detail. A total of 190 isolates from MRS- and LBS-agar plates were purified, however, only 66 of the isolates were Gram+ and catalase-negative and presumably LAB. This level agrees with O'Sullivan et al. (1999) observing that in a modern malthouse less than 10% of the total microbial load was identified as LAB. They described the dominant microflora during malting to be Gram-negative pseudomonads. In our study, the dominating genus among the Gram+ and catalase-negative isolates were *Leuconostoc* being characterized as coccoid-rod shaped bacteria (57). A selection of the isolates was identified by 16S rRNA sequencing as *Leuconostoc citreum/holzapfelii* (20) and *Leuconostoc pseudomesenteroides/mesenteroides* (3). The minor LAB population (9%) was rod shaped and belonged to *Carnobacterium*. Booysen et al. (2002) isolated and identified 67 LAB throughout the malting of two barley cultivars and 38 were identified as *Leuconostoc lactis* and *Leuconostoc argentinum*. This agrees with the observation of O'Sullivan et al. (1999) too, who observed that the majority of LAB is dominated by mesophilic heterofermentative cocci.

As mentioned, growth of moulds was considerably lower on the MRS-plates with germinated samples. An increase in LAB during germination will cause production of organic acids from the fermentation of carbohydrates. Only lactic acid will be produced if the LAB flora mainly consists of homofermentative LAB, and lactic acid, ethanol, acetic acid and CO_2 if the LAB flora is dominated by heterofermentative LAB, however, both organic acids will cause inhibition of the moulds. A dramatic increase in LAB species belonging to the genera *Lactobacillus*, *Lactococcus* and *Leuconostoc* were seen at the germination step,

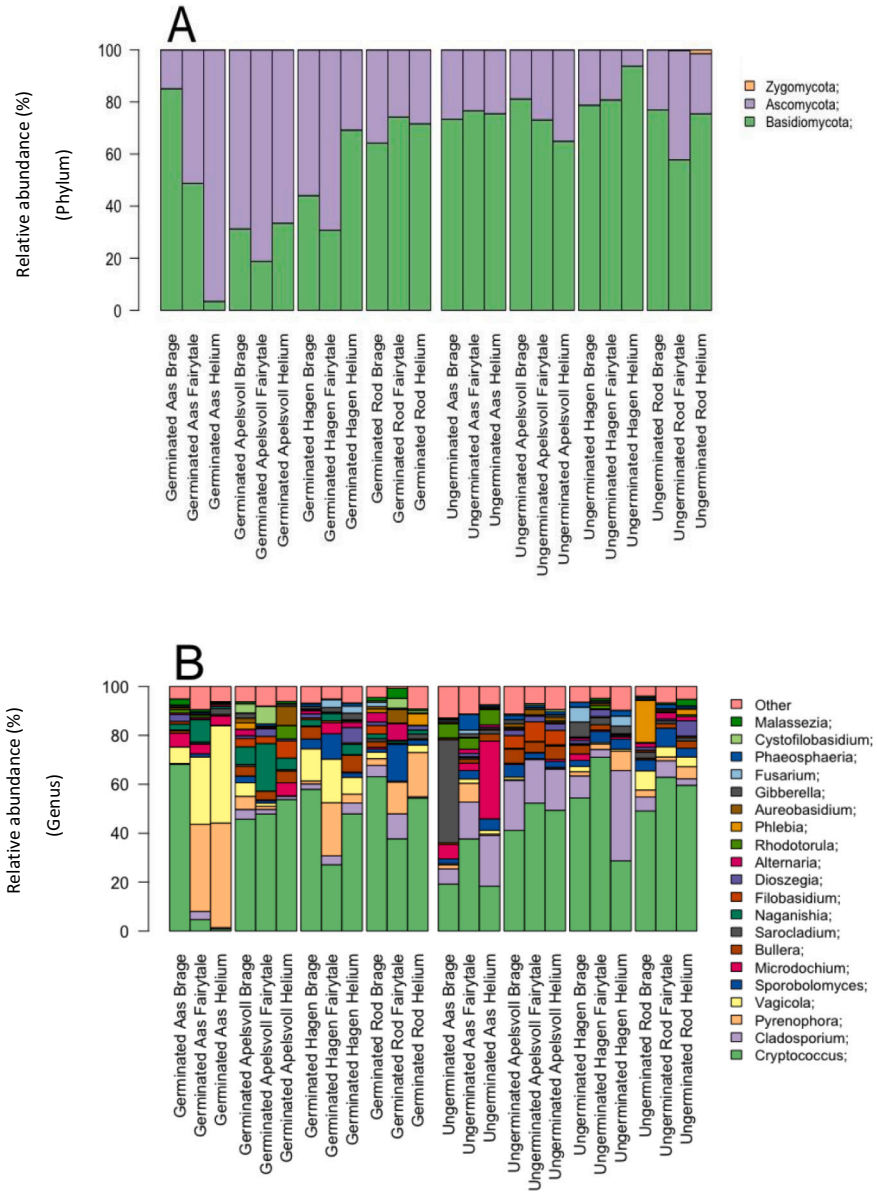

Fig. 3. Relative abundance of (A) fungal phyla and (B) the 20 most representative fungal genera in the samples of germinated and ungerminated barley cultivars cultivated in four geographical regions in Norway. Other: indicate genera with relative abundance <0.05%.

indicating their important role during malting (Justé et al., 2014; Li et al., 2018). On LBS agar no growth was detected from the ungerminated samples, except for one sample, however, on germinated barley samples from Aas and Hagen some growth was seen. Østlie et al. (2004, 2005) found that the selectivity of the LBS agar regarding lactobacilli was relatively good with 80–83% of the colonies identified as *Lactobacillus* sp. A reason for the low growth results on the LBS-agar can be low levels of lactobacilli in the LAB population. In this study, there seem to be a 100% domination of *Leuconostoc* in germinated samples from Helium grown at Aas and Fairytale grown at Hagen regarding the isolates from LBS agar. However, from the culture independent high-throughput sequencing analysis only the *Lactococcus* LAB genus was seen among the 20 most representative bacterial genera in the samples.

The microbial community of Norwegian barley cultivars studied by culture independent high-throughput sequencing showed significant differences in bacterial diversity in ungerminated and germinated barley samples, while no significant differences in bacterial composition were detected between the cultivar and the place. The germination process increased the diversity of bacterial genera having a relative abundance greater than 1%. In total, 18 genera (with abundance greater than 1%) were found in germinated samples and 9 in ungerminated samples. Conditions that enable grain germination also favour growth of microorganisms. The increase in humidity and also the germination temperature will activate dormant psychrotrophic and mesophilic bacteria (Laitila et al., 2007).

In the study of Li et al. (2018), both bacteria and fungi were identified during the malting process by culture dependent and independent methods. In the barley samples based on culture dependent methods, only *Citrobacter* and *Enterobacter* bacterial genera were identified, however, in the green malt a much larger diversity of genera were found as *Bacillus*, *Citrobacter*, *Escherichia coli*, *Kluyvera*, *Pantoea*, and *Photorhabdus*. By the culture-independent PCR-denaturing gradient gel electrophoresis (PCR-DGGE) method three PCR-DGGE bands were seen in the ungerminated samples where only *Kluyvera* was identified from one of the bands, indicating low bacterial diversity, however, during steeping and germination a large increase in PCR-DGGE bands (15) were seen (Li et al., 2018). These PCR-products were classified as members of the genera *Erwinia*, *Lactobacillus*, *Leuconostoc*, *Wautersiella*, *Pantoea*,

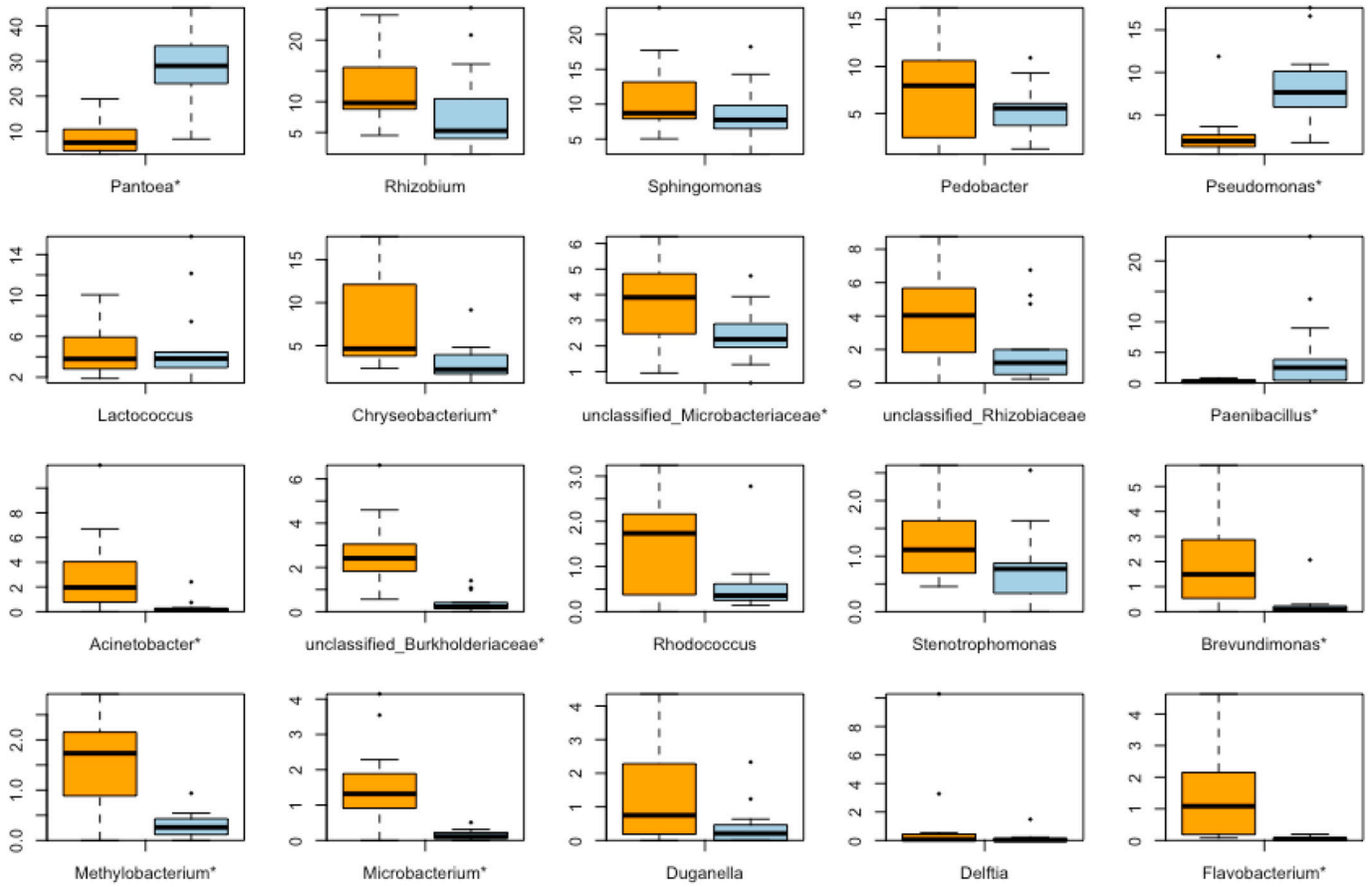

Fig. 4. Relative abundance distribution (%) of the 20 most represented bacterial genera in germinated (orange) and ungerminated (light blue) samples. *: indicates significant differences between the 2 type of samples calculated using the Mann-Whitney test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Flavobacterium, *Acinetobacter*, *Enterobacter*, *Lactococcus*, *Streptococcus* and *Kluyvera*. The most abundant bacterial sp. in the ungerminated and germinated samples in our study was *Pantoea*, being more abundant in the ungerminated samples than in the germinated one, followed by *Rhizobium* and *Sphingomonas*. *Pantoea* is a diverse group of rod-shaped Gram-negative bacteria in the *Enterobacteriaceae* family, widely distributed in nature. The genus includes several species generally recognized as plant pathogens, but some species can cause disease in humans (Delétoile et al., 2009; Walterson and Stavrinides, 2015). The reduction of bacteria belonging to the *Pantoea* genus during germination is positive both from a plant perspective, but also from a human pathogen perspective. *Rhizobium* is a Gram-negative soil bacterium that fixes nitrogen found at root nodules of legumes. Many legume species are used as soil-enriching green manure due to their ability to fix atmospheric N_2 through rhizobia living symbiotically on the plant roots (Ntatsi et al., 2018). Many farmers grow legumes in crop rotation to increase the yield of grain crops. In addition, among the 20 most representative bacterial genera, *Flavobacterium*, *Acinetobacter* and *Lactococcus* were also found with *Flavobacterium* and *Acinetobacter* being more abundant in the germinated samples and *Lactococcus* showing about the same abundance both in ungerminated and germinated samples. Li et al. (2018) found the Gram-negative bacteria *Pantoea agglomerans* and *Pantoea* sp., *Flavobacterium* sp., *Acinetobacter* sp. and *Enterobacter* dominating the bacterial community in the samples during the malting process. A large number of Gram-negative bacteria like *Acinetobacter*, *Erwinia*, *Enterobacter*, *Kluyvera* and *Pantoea* may negatively affect the malting process and cause severe mash filtration difficulties (Li et al., 2018). Many of the mentioned genera, such as *Erwina*, *Enterobacter*, *Pseudomonas*, *Pantoea* and *Rahnella* species, were also found to dominate the bacterial community during the malting process by Laitila et al. (2007).

Fungal composition of the germinated and ungerminated grain samples was significantly influenced by the germination process and the cultivation place of the grains. The culture independent high throughput sequencing results of fungi showed the estimated number of operational taxonomic units (OTUs) to be higher in the ungerminated samples than in the germinated samples, however, the diversity in the samples was the same. This is opposite of the bacterial results, showing higher estimated number of OTUs and a higher diversity in the germinated samples. The most abundant fungi in both ungerminated and germinated Norwegian barley samples were *Cryptococcus*, *Cladosporium* and *Pyrenophora*. High abundance of *Cryptococcus* was seen in all samples except germinated samples of Fairytale and Helium from Aas. *Cryptococcus* is a commonly found Basidiomycota yeast in barley grains (Korhola et al., 2014; Noots et al., 1999; Olstorpe et al., 2010). *Cryptococcus flavescens* is an antagonist of Fusarium head blight caused by *Fusarium graminearum* in wheat and barley causing significant losses of yield and quality worldwide (Schisler et al., 2002, 2014; Zhang et al., 2007). Higher abundance of *Pyrenophora* was seen in the germinated samples, especially in samples from Aas (all cultivars except Brage), Fairytale from Hagen, and Fairytale and Helium from Rød compared to the other germinated samples, but also compared to the ungerminated samples. However, the ungerminated samples always contained some *Pyrenophora* except samples from Apelsvoll. *Pyrenophora* consists of many different species, however, *Pyrenophora teres* is a common plant pathogen that causes net blotch on barley (Liu et al., 2011). It is a disease that is distributed worldwide and is particularly prevalent in cold and wet growing regions. In Norway the fungus can cause yield losses of up to 40% under conducive conditions

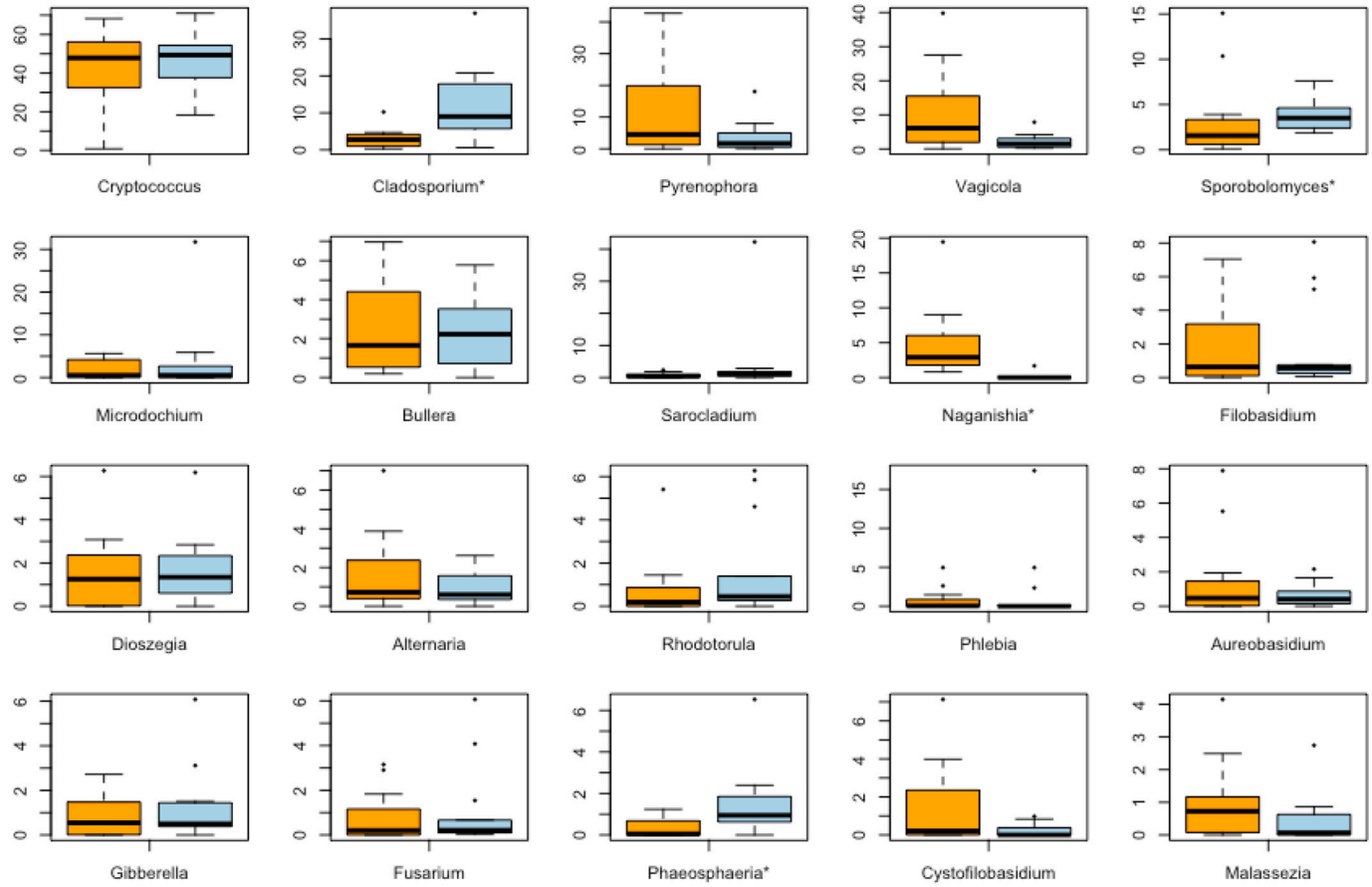

Fig. 5. Relative abundance distribution (%) of the 20 most represented fungal genera in germinated (orange) and ungerminated (light blue) samples. *: indicates significant differences between the 2 type of samples calculated using the Mann-Whitney test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Wonneberger, 2017). Climatic differences may explain the observed differences in *Pyrenophora* at the different farms. *Pyrenophora* seem to be a common fungus being present especially in germinated samples (Li et al., 2018). *Cladosporium* showed higher abundance in ungerminated samples and was clearly seen in all ungerminated samples and *Alternaria* was seen in 42% of the ungerminated samples and in 70% of the germinated samples. *Cladosporium* and *Alternaria* are common field fungi that require relative high-water activities for optimal growth, and they are well adapted to the sometimes rapidly changing temperatures that can occur during a sunny day and the cool nights (Adams et al., 2016). *Epicoccum* was not seen and *Fusarium* only in 25% of both ungerminated and germinated samples among the 20 most representative fungal genera in this study, even though they are common fungi found in the field and both in the field and during storage, respectively. Chen et al. (2016) observed that the relative abundance of *Fusarium* and *Alternaria* significantly increased and *Cladosporium* significantly decreased through the malting process, but Justé et al. (2011) observed that *Cladosporium* and *Alternaria* normally decreased during germination. Li et al. (2018) observed an increase in the fungi load during the malting, however, the variation in fungi diversity was less than the variation in bacterial diversity. In the barley samples three genera of yeasts belonging to *Cryptococcus*, *Rhodotorula* and *Sporobolomyces* and three genera of filamentous fungi belonging to *Aspergillus*, *Mucor* and *Rhizopus* were identified by culture dependent methods. At the end of germination nearly the same genera were seen except *Sporobolomyces*. *Sporobolomyces* was seen in all ungerminated samples of our study too, but not in the all germinated samples.

The germination process affected both bacterial and fungal microbiota composition in this study, however, no difference in bacterial microbiota were detected between cultivars or cultivation place. *Pantoea* was the most abundant bacterial sp. both in ungerminated and germinated barley samples. The fungal community was significantly affected by the cultivation place. The most abundant fungi in both ungerminated and germinated Norwegian barley samples were *Cryptococcus*, *Cladosporium* and *Pyrenophora*. This agrees with Kaur et al. (2015) that studied fungal and bacterial community structure of barley malts from diverse geographical regions around the world and found that differences in fungal communities of malts of different geographical location were more pronounced than differences in bacterial communities. Kaur et al. (2015) also described that the differences in fungal communities of malts were more important than the bacterial communities when it came to influencing the quality of the malt.

The use of LAB is one of the oldest form of food preservation and today they are still in use in many fermented foods. The utilization of LAB cultures might inhibit deleterious Gram-negative aerobes, yeasts and moulds during malting and brewing (Lowe and Arendt, 2004; Laitila et al., 2006a). In a study by Laitila et al. (2006a) it was concluded that LAB starter cultures had many beneficial effects on the malt quality. The starter cultures restricted growth of harmful bacteria and *Fusarium* sp. Many *Fusarium* sp. are causal agents of important plant diseases and/or are agents of mycotoxin producers. Some *Fusarium* sp. are also known to produce hydrophobins linked to gushing (Chen et al., 2016). The isolated LAB belonged to the genera *Carnobacterium*, *Lactococcus*, *Enterococcus* and *Leuconostoc*. These isolates might be used as starter cultures in sour beer production. In addition, their antimicrobial potential regarding inhibition of potentially deleterious Gram-negatives and fungi related to malting and brewing can be tested. *Leuconostoc* are capable of EPS production which may cause severe processing and quality

problems. In general, LAB can be used as a natural food-grade biocontrol agents.

The cultivation place and climate are important related to the quality of malt and subsequently also the beer. Knowledge of the microbial communities of the germinated barley will enhance our understanding of the malting process and may lead to enhanced process control and production of malt of high quality. LAB was isolated from germinated Norwegian barley and their functionality related to malting and brewing will be tested. Several metabolic properties of LAB can have an impact on safety and flavour during malting and brewing.

5. Conclusions

The microbial community of ungerminated and germinated barley grains from three different cultivars grown at four different locations in Norway was identified by culture dependent and culture independent methods. One focus was to isolate LAB from the barley grains. Germination significantly influenced the bacterial and fungal microbiota composition. The most abundant bacterial genera were *Pantoea*, *Rhizobium* and *Sphingomonas* and fungal genera were *Cryptococcus*, *Cladosporium* and *Pyrenophora*. Regarding influence from different cultivars and growth places on bacterial and fungal compositions only the cultivation place significantly influenced the fungal composition. Few LAB were isolated from the germinated barley samples (66 out of 190 isolates). The dominating isolates belonged to *Leuconostoc* sp., and especially the species *Leu. citreum/holzapfelii* was dominating. Minor species identified were *C. aromaticum*, *L. lactis* and *Enterococcus*. The isolated LAB might have the potential as starter cultures in sour beer production. It is not known if the LAB species identified in this study will influence the yeast fermentation, the organoleptic properties of beer or cause spoilage of the beer. The fermentative ability of some of the isolated LAB either as single strains or as bacterial consortium with and without yeast is being tested in wort and in a variant of sour beer.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2021.109059.

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Declaration of competing interest

The authors have no competing interest to declare.

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