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Microdochium majus and other fungal pathogens associated with reduced gluten quality in wheat grain

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ABSTRACT

The bread-making quality of wheat depends on the viscoelastic properties of the dough in which gluten proteins play an important role. The quality of gluten proteins is influenced by the genetics of the different wheat varieties and environmental factors. Occasionally, a near complete loss of gluten strength, measured as the maximum resistance towards stretching (R_{max}), is observed in grain lots of Norwegian wheat. It is hypothesized that the loss of gluten quality is caused by degradation of gluten proteins by fungal proteases. To identify fungi associated with loss of gluten strength, samples from a selection of wheat grain lots with weak gluten (n = 10, R_{max} < 0.3 N) and strong gluten (n = 10, R_{max} ≥ 0.6 N) was analyzed for the abundance of fungal operational taxonomic units (OTUs) using DNA metabarcoding of the nuclear ribosomal Internal Transcribed Spacer (ITS) region ITS1. The DNA quantities for a selection of fungal pathogens of wheat, and the total amount of fungal DNA, were analyzed by quantitative PCR (qPCR). The mean level of total fungal DNA was higher in grain samples with weak gluten compared to grain samples with strong gluten. Heightened quantities of DNA from fungi within the Fusarium Head Blight (FHB) complex, i.e. *Fusarium avenaceum*, *Fusarium graminearum*, *Microdochium majus*, and *Microdochium nivale*, were observed in grain samples with weak gluten compared to those with strong gluten. *Microdochium majus* was the dominant fungus in the samples with weak gluten. Stepwise regression modeling based on different wheat quality parameters, qPCR data, and the 35 most common OTUs revealed a significant negative association between gluten strength and three OTUs, of which the OTU identified as *M. majus* was the most abundant. The same analysis also revealed a significant negative relationship between gluten strength and *F. avenaceum* detected by qPCR, although the DNA levels of this fungus were low compared to those of *M. majus*. In vitro growth rate studies of a selection of FHB species showed that all the tested isolates were able to grow with gluten as a sole nitrogen source. In addition, proteins secreted by these fungi in liquid cultures were able to hydrolyze gluten substrate proteins in zymograms, confirming their capacity to secrete gluten-degrading proteases. The identification of fungi with potential to influence gluten quality can enable the development of strategies to minimize future problems with gluten strength in food-grade wheat.

1. Introduction

Common wheat (*Triticum aestivum*) is one of the most important cereals used for bread-making. When wheat flour is mixed with water, gluten proteins form a continuous network that confers the viscoelastic properties to dough which are necessary for baking bread (Shewry et al., 1995). Gluten proteins are classified into two main groups, monomeric gliadins that affect dough viscosity, and polymeric glutenins that give dough its elasticity and strength (Goesaert et al., 2005; Shewry et al., 1995). The largest and least soluble glutenin polymers, which are enriched in high molecular weight glutenin subunits, are positively correlated with dough strength (Gupta et al., 1993).

The bread-making quality of wheat is the product of genetics of wheat varieties combined with the effect of environmental factors. Elasticity or dough strength is known to be strongly connected to the allelic variation in the high molecular weight glutenin subunits that differ between varieties (Payne et al., 1979). Environmental factors, such as temperature and the availability of water and nutrients during

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plant and kernel development and maturation, influence the accumulation and composition of gluten proteins (Altenbach, 2012). The details of how environmental factors affect gluten quality, however, are not fully understood. In Norway, wheat grain lots are graded at delivery according to a classification system for wheat varieties by their potential gluten strength, determined in variety trials established for the testing and release of new varieties. Thereby the variation in gluten quality caused by environmental factors is not accounted for at grain delivery. This causes unpredictable variation within and between the wheat quality classes and challenges the miller's ability to consistently produce quality flour for the baking industry.

Microorganisms, including fungi, can influence gluten quality in wheat. Wheat, both naturally and artificially infected with *Fusarium* spp. during plant development, has been shown to yield flour with poor dough performance and reduced loaf volume when baked (Dexter et al., 1996; Koga et al., 2019b; Nightingale et al., 1999; Wang et al., 2005). *Fusarium* infection has been reported as having only a minor effect on the total protein content of wheat (Dexter et al., 1996; Eggert et al., 2010; Wang et al., 2005), however changes in gluten protein composition have been observed in infected compared to uninfected kernels. In infected kernels, reduced glutenin fractions have been measured in combination with stable or increased gliadin fractions. Dexter et al. (1996) proposed that a possible explanation for the change in gluten protein composition is that *Fusarium*, by causing early death of infected kernels, reduces glutenin synthesis. Others have shown that *Fusarium* invades the wheat endosperm, where it degrades storage proteins and starch (Jackowiak et al., 2005). Likewise, Nightingale et al. (1999) and Wang et al. (2005) speculated that *Fusarium*-infected wheat kernels may contain fungal proteases capable of degrading gluten during processing, thus resulting in loss of gluten functionality. This was recently investigated in more depth by confirming the presence of gluten degrading proteases in wheat grain harvested from a *F. graminearum* inoculated field (Koga et al., 2019a, 2019b).

The gluten quality of Norwegian bread wheat has been analyzed since 2005 by using a method in which washed gluten is stretched on a Kieffer dough and gluten extensibility rig to measure maximum resistance to streching (R_{max}) and extensibility. The data reveals considerable differences in gluten quality due to the environmental conditions during the growing season (Koga et al., 2016b; Moldestad et al., 2011). Particularly poor gluten quality has been observed in grain harvested from fields where the period of grain filling was characterized by low temperatures and frequent rainfall events. Further investigations indicated the presence of gluten-degrading proteases in grain samples with poor gluten quality that was coincident with a high incidence of *Fusarium* and *Microdochium*, suggesting that the proteases may be of fungal origin (Koga et al., 2016b).

Wheat grain usually hosts a large number of fungi, known as the wheat grain mycobiota, from which gluten-degrading proteases may originate. Some of these fungi are known wheat pathogens, including *Fusarium* spp., *Microdochium* spp. and *Parastagonospora nodorum*, whereas others are principally saprophytic or surface contaminants of the grain. Methods that have been developed for studying the wheat grain mycobiota include grow out tests/plate counts, blotter tests, or diagnostic PCRs. These methods have certain limitations in their abilities to give a complete picture of the mycobiota, as grow outs and blotter tests are limited to the detection of fungi that thrives on the test medium and/or that are alive at the time of testing, whereas diagnostic PCRs are limited to target species. To obtain a more comprehensive insight of the mycobiota of plants, methods for microbial community profiling have been adopted (Schlaeppi and Bulgarelli, 2015). DNA metabarcoding aims to analyze all microbes associated with plants at the DNA level by high throughput sequencing of microbial barcodes in environmental samples. Recent studies have utilized this method to study the wheat grain mycobiome, revealing the possibility of a detailed picture of the fungal community, including pathogens, saprophytes, yeasts and other fungi (Hertz et al., 2016; Links et al., 2014; Nicolaisen et al., 2014; Yuan et al., 2018). Studies have focused on changes in the fungal community during wheat head development (Hertz et al., 2016) or grain storage (Yuan et al., 2018), as well as identification of fungal coexistence patterns (Nicolaisen et al., 2014) and antagonistic relationships (Links et al., 2014). To our knowledge, metabarcoding of the fungal communities in wheat grain and studies of the association of fungal communities with wheat gluten quality have not previously been conducted.

We hypothesize that the near complete loss of gluten strength occasionally observed in grain lots of Norwegian wheat is partly caused by a degradation of gluten proteins by fungal proteases. Therefore, the aims of this study were to i) identify fungi associated with reduced gluten quality in Norwegian wheat using metabarcoding and species-specific qPCR, and ii) test a number of these fungi for their ability to break down or utilize gluten in vitro on gluten-amended media and with zymography.

2. Material and methods

2.1. Wheat samples with different gluten strength

Wheat grain samples with either strong gluten (n = 10) or weak gluten (n = 10) were selected from materials used for the annual quality assessment of spring and winter wheat from 2011 to 2014 (Table 1). Gluten strength was measured as the maximum resistance towards stretching (R_{max}) using the Kieffer Extensibility rig (Kieffer et al., 1998), and was categorized as weak when the R_{max} values were lower than 0.3 N, and strong when the R_{max} values were over 0.6 N. The trials, conducted at multiple locations, were sited in commercial fields as a complete, randomized block design with two replicates. Management practices were similar to those used for commercial fields with respect to tillage, fertilization, and weed control. Fungicides were applied as follows: In the spring wheat trials, Stereo 312.5 EC (150 ml/daa, Syngenta Crop Protection AG, Basel, Switzerland, active ingredients cyprodinil [23.8% W/W] and propiconazole [5.9% W/W]) was applied at growth stage BBCH 37 (flag leaf just visible; (Lancashire et al., 1991)) and Proline EC 250 (80 ml/daa, Bayer Crop Science AG, Monheim, Germany, active ingredient prothioconazole [251 g/l]) at BBCH 55. In winter wheat, Stereo 312.5 EC (150 ml/daa) was applied at BBCH 31, and Proline EC 250 (60 ml/daa) and Delaro SC 325 (30 ml/daa, Bayer Crop Science AG, active ingredients trifloxystrobin [150 g/l] and prothioconazole [170 g/l]) at BBCH 55. The fields were harvested in August/September. Harvested plots normally yielded 4–7 kg of grain. A 1 kg sample was collected immediately from the harvest, dried in a drying chamber until moisture was below 15%, and cleaned. Protein content was analyzed using near infrared transmittance using a Foss Infratec™ 1241 Grain Analyzer (FOSS Tecator AB, Höganes, Sweden). A 200 g sub-sample of grain was milled into whole-meal flour in a Laboratory Mill 3100 (Perten Instruments AB, Huddinge, Sweden) with a 0.8 mm screen. The flour was stored at room temperature for at least two weeks, then analyzed for falling number (AACCI Method 56-81.03), and gluten quality with the Kieffer Extensibility rig (Table 1). The remaining grain was stored in a refrigerator at 3 °C until 2015, when it was milled to whole-meal flour as described above and stored at −20 °C until mycotoxin (deoxynivalenol) analysis and DNA extraction were performed.

2.2. DNA extraction

Total genomic DNA was extracted from 100 mg of flour using a FastDNA SPIN Kit for Soil (MP Biomedicals, Solon OH, USA) following the manufacturers' directions. The quality of the DNA was assessed using agarose gel electrophoresis and quantified using a Nanodrop spectrophotometer 2000 (Thermo Scientific, Wilmington, DE, USA).

Table 1
Grain samples of Norwegian spring or winter wheat harvested from field trials conducted at multiple locations from years 2011 to 2014 and analyzed in this study.

Sample	Spring/winter wheat	Year grown	Municipality, County	Cultivar	R_{max} (N)[a]	Falling number (s)[b]	Total protein (%)[c]	DON (μg/kg)[d]
A	Winter	2011	Østre Toten, Oppland	Finans	0,12	236	9,2	< 250
B	Winter	2012	Re, Vestfold	Finans	0,13	335	12,4	< 200
C	Winter	2011	Nes, Akershus	Olivin	0,14	244	13,3	1128
D	Winter	2014	Ullensaker, Akershus	Finans	0,16	404	12,2	< 200
E	Spring	2012	Nes, Akershus	Zebra	0,16	322	11,5	344
F	Winter	2011	Nes, Akershus	Olivin	0,18	233	10,7	1391
G	Spring	2012	Nes, Akershus	Bjarne	0,2	278	12,1	386
H	Spring	2013	Holmestrand, Vestfold	Bjarne	0,21	326	11,6	< 200
I	Spring	2012	Nes, Akershus	Krabat	0,21	310	13,1	749
J	Spring	2012	Nes, Akershus	Zebra	0,22	334	12,3	339
K	Spring	2012	Holmestrand, Vestfold	Zebra	0,6	338	10,8	< 200
L	Winter	2011	Hole, Buskerud	Finans	0,63	369	11,9	< 200
M	Spring	2012	Holmestrand, Vestfold	Bjarne	0,68	381	11	< 200
N	Spring	2012	Holmestrand, Vestfold	Zebra	0,69	360	10,7	< 200
O	Spring	2012	Holmestrand, Vestfold	Krabat	0,72	377	10,7	254
P	Winter	2014	Ullensaker, Akershus	Olivin	0,73	433	12,4	< 200
Q	Winter	2011	Hole, Buskerud	Olivin	0,75	412	11,7	< 200
R	Winter	2011	Hole, Buskerud	Finans	0,80	379	11,9	< 250
S	Spring	2013	Holmestrand, Vestfold	Bjarne	0,80	308	12	< 250
T	Winter	2012	Stange, Hedmark	Olivin	0,86	319	11,2	291

[a] The maximum resistance towards stretching of gluten (R_{max}), i.e. the force (N) used to stretch gluten pellet measured by Kieffer Dough and Gluten Extensibility Rig (Kieffer et al., 1998).
[b] Falling number (s) measured using AACCI Method 56-81.03.
[c] Total protein content (%) measured by near infrared transmittance using Foss Infratec™ 1241 Grain Analyzer (FOSS Tecator AB, Höganes, Sweden).
[d] Deoxynivalenol (DON) measured with ELISA (AgraQuant® Deoxynivalenol Assay).

2.3. Microbial profiling by sequencing (metabarcoding)

For the genomic DNA from our samples, Illumina libraries were prepared using ITS1 primers and the protocol from www.earthmicrobiome.org (ITS1f-ITS2, EMP.ITSkabir). The resulting PCR products were purified using the Agencourt XP Ampure Beads (Beckman Coulter Inc., Brea CA, USA), and sequenced using MiSeq Reagent Kit v3 (600-cycle) on an Illumina MiSeq. Fungal sequences were processed and analyzed using a customized bioinformatics pipeline (Song et al., 2017). The sequences were clustered into operational taxonomic units (OTUs) using 97% similarity threshold and classified using UNITE fungal ITS database (sh_general_release_dynamic_01.12.2017). Since the UNITE fungal database currently do not fully cover all fungi associated with wheat heads, we also included sequences from our NIBIO plant pathogen isolate collection to obtain a better taxonomic resolution (Table S1, Appendix 1). These isolates were identified to species based on morphology and the identities confirmed by ITS Sanger sequencing (White et al., 1990). The BOLD Identification System for ITS was used for confirming the UNITE classification for the 35 most common OTUs.

The degree of diversity in the microbial communities was calculated as Inverse Simpson (1/D) using Mothur v1.40.5. The β-diversity was calculated as the distances between the microbial communities (Thetayc in Mothur v1.40.5). The distances were visualized using tools from Interactive Tree Of Life (Letunic and Bork, 2006).

The Mann-Whitney test was used to assess whether the number of OTUs or the community diversity were equal or different among the two groups of wheat grain samples (weak vs. strong gluten). Spearman rank correlations were used to assess the correlation between the total level of fungal DNA and the number of OTUs, or the community diversity. Levene's test were used to assess whether the variance in number of OTUs or community diversity were equal or different between the two groups of samples. All tests were conducted in Minitab 18.

2.4. Quantification of DNA of selected fungal species (qPCR)

Total genomic DNA extracted from our samples was analyzed with qPCR to quantify DNA from eight common fungal wheat pathogens: *F. avenaceum*, *Fusarium culmorum*, *F. graminearum*, *Fusarium poae*, *Fusarium sporotrichioides, M. majus, M. nivale,* and *P. nodorum*. In addition, the host plant and total fungal DNA were quantified in each sample. The probes and/or primers used are described in Table S2 (Appendix 1). Assays for *M. nivale* and *P. nodorum*, and total fungal DNA were SYBR Green assays, all others were probe assays.

The qPCR using probe assays was performed according to Hofgaard et al. (2016b) in a total volume of 25 μl, consisting of 4 μl genomic DNA from wheat samples (diluted 1 + 9 with PCR grade water) or DNA from pure cultures (standards), 300 nM of each primer, 100 nM of each probe, and 1× Sso Advanced™Universal Probes Supermix, (Bio-Rad, Hercules, CA, USA), in a C1000 Touch Term Cycler combined with a CFX96TM Real-Time System (Bio-Rad). In the current study, *F. avenaceum* and *F. culmorum* were combined to duplex reactions consisting of 300 nM forward- and 100 nM reverse-primer, 100 nM probe, and iQ™ Multiplex Powermix (Bio-Rad). SYBR assays were performed using 1× Sso Advanced™ Universal SYBR® Green Supermix (Bio-Rad).

Genomic DNA from pure cultures of the different fungi was extracted according to the protocol of Koga et al. (2019a). For quantification of DNA from the different fungi, five serial dilutions in the range 1–4000 pg of DNA from pure cultures of the respective species were used. For the quantification of host plant DNA, the serial dilution contained plant DNA in the range 0.08–32 ng. The amount of fungal DNA was normalized against the amount of plant DNA, and fungal content was presented as pg fungal DNA per ng plant DNA (pg/μg).

2.5. Analysis of deoxynivalenol

The samples were analyzed for the mycotoxin deoxynivalenol using an ELISA method (AgraQuant® Deoxynivalenol Assay, Romer Labs® Tulln, Austria). Sample extracts were made by adding 25 ml of distilled water to 5 g of ground sample, followed by vigorous shaking for 3 min. The mixture was centrifuged for 1 min at 1811*g*, and the supernatant was diluted 1 + 3 with distilled water. The ELISA analysis was performed on the diluted supernatant according to the manufacturer's instructions.

2.6. Associations between gluten strength and fungal content

Potential associations between gluten strength and different quality parameters were initially investigated using a General Linear Model (GLM in Minitab 18). The response variable was gluten strength (R_{max}), the fixed factors were year and winter/spring wheat, and the following covariates: falling number, protein level, deoxynivalenol content, and quantity of total fungal DNA, as well as all possible interactions between the different factors. The covariates were standardized by using the function in GLM «Subtracting the mean, then divide by the standard deviation». We also tried a second model that was identical to the first, except that wheat variety was included as a fixed factor instead of winter/spring wheat. In addition, we also explored possible associations between gluten strength and DNA content of the different fungal species measured by qPCR. For this, we used stepwise regression models in Minitab 18, with the fixed factors year and winter/spring wheat (or year and wheat variety), and the possible covariates falling number, protein level, deoxynivalenol content, and level of DNA measured by qPCR from the following fungal species: *F. avenaceum*, *F. graminearum*, *M. majus*, *M. nivale*, and *P. nodorum*.

Finally, we used stepwise regression analyses in Minitab 18 to assess possible associations between gluten strength, and the continuous predictors: wheat quality parameters (falling number, total protein, deoxynivalenol), DNA content of fungal species detected by qPCR, total fungal DNA (qPCR), and the abundancy (number of sequences) of 35 of the most common OTUs detected by metabarcoding. The categorical predictors included were year and winter/spring wheat (or year and wheat variety). OTUs with > 1000 sequences in total across all samples were included. The fixed factors in the model were year and winter/spring wheat (or year and wheat variety). The continuous predictors were standardized by choosing the option «Subtract the mean, then divide by the standard deviation». Predictors with a variance inflation factor (VIF) > 5 were excluded from the final models.

2.7. Growth of selected fungi on different nitrogen sources

Three isolates each of *F. avenaceum*, *F. graminearum*, *M. majus*, and *M. nivale*, previously isolated from Norwegian cereals or grasses (Table 2), were grown on each of four media types containing different nitrogen sources. Three of the media included minimal media (Leslie and Summerell, 2006) supplemented with only one of the following nitrogen sources each: sodium nitrate ($NaNO_3$, 2 g/l), gluten from wheat (Sigma-Aldrich, St. Louis MO, USA; 2.4 g/l), or N-Z Amine® A (Sigma-Aldrich; 3 g/l). Bacto™ Agar (Difco, Laboratories, Detroit MI, USA; 20 g/l) was added as a solidifying agent. The final medium was a complete medium (CM) that contained nitrogen from $NaNO_3$, casein/N-Z Amine, and yeast extract, meant to facilitate growth in all fungal isolates (Leslie and Summerell, 2006). All four growth media were standardized to contain the same amount of nitrogen.

Mycelial plugs of the selected fungi were taken from −80 °C storage and transferred to potato dextrose agar (PDA) medium in Petri dishes, placed in the dark at 9 °C for six days, and then incubated on the lab bench at room temperature for four days. The amount of PDA medium in the Petri dishes was reduced to roughly half the usual amount to minimize transfer of PDA medium with new mycelium plugs. Mycelium plugs approximately 5 mm in diameter, were punched from the colony margin and transferred to Petri dishes containing 25 ml of the growth medium to be tested. Each isolate-medium combination had a total of three replicates (i.e. plates) in the experiment. The plates were incubated in the dark at 15 °C for eight days. Mycelial growth (mm) on each of the media was registered daily from day three to eight by marking growth along four radii on the underside of the Petri dish. Mycelial growth rates were determined by measuring the distance between the marks and calculating the average of the four measurements per plate.

Possible relationships between fungal species, and growth medium with the observed growth rates were assessed in Minitab 18 using a GLM with Fisher LSD for pairwise comparisons. The model included the response variable of mycelial growth rates measured from day three to six, the fixed factors of fungal species and growth medium, and the interaction of fungal species and growth medium. Data were transformed using the option λ.

Table 2
Accession information for isolates of *Fusarium avenaceum*, *Fusarium graminearum*, *Microdochium majus*, *Microdochium nivale*, and *Parastagonospora nodorum* isolated from plants grown in Norway, and used in the study of growth rate on different media, and/or the study of protease activity (zymography).

Species	Isolate[a]	Year[b]	Host	In vitro test
F. avenaceum	201 030	2004	Wheat, grain	Growth rate, protease activity
F. avenaceum	201 063	2005	Wheat, leaf	Growth rate
F. avenaceum	202 021	2012	Wheat, straw	Growth rate
F. graminearum	200 630[c]	2006	Wheat	Growth rate, protease activity
F. graminearum	201 196[c]	2012	Wheat, straw	Growth rate
F. graminearum	202 058[c]	2013	Wheat, grain	Growth rate
M. majus	200 345	2003	Wheat, grain	Growth rate
M. majus	200 417	2003	Wheat, grain	Growth rate
M. majus	200 430	2004	Wheat, leaf	Growth rate, protease activity
M. nivale	200 231	1996	Ryegrass	Growth rate
M. nivale	200 272	1999	Meadow festuce	Growth rate, protease activity
M. nivale	201 050	2010	Festulolium	Growth rate
P. nodorum	201 204	–	–	Protease activity

[a] NIBIO isolate number.
[b] Year of isolation.
[c] Further information about isolates are provided in Aamot et al. (2015).

2.8. Gluten-degrading ability of selected fungi (zymography)

To assess their gluten-degrading ability, one isolate each of *F. avenaceum*, *F. graminearum*, *M. majus*, *M. nivale*, and *P. nodorum* (Table 2) were grown in liquid cultures containing the complete medium or the minimal medium amended with gluten. The media were prepared as described above. Mycelial plugs of fungal cultures were taken from −80 °C storage, transferred to Petri dishes containing complete medium or minimal medium with gluten, both solidified with agarose. The cultures were incubated in the dark at 18 °C. When the fungal growth approached the outer edge of the plates (six days for *Fusarium* spp. and *Microdochium* spp., eleven days for *P. nodorum*), the agar with mycelium was cut into small pieces and transferred to 500 ml Erlenmeyer flasks containing 150 ml liquid formula (without agarose) of the media on which the isolate had been incubated. The liquid cultures were incubated in the dark at 18 °C for seven days.

Following the incubation period cultures were filtered through two layers of gauze and Whatman filter paper No.1 (GE Healthcare, Amersham, Buckinghamshire, UK), and the filtrates were centrifuged at 39,200*g* for 20 min to remove excess particles. A total of 40 ml supernatant per unit was concentrated with an Amicon Ultra-15 Centrifugal Filter Unit 3 K (Merck Millipore, Darmstadt, Germany) using a TJ-25 centrifuge (Beckman Coulter) with a swing rotor TS-5.1-500 at 4000*g* for 60 min at 4 °C. The protein concentration was measured by a Lowry protein assay (Bio-Rad), and kept at −80 °C until further use.

The presence of fungal proteases in the concentrated supernatants and their ability to hydrolyze gluten proteins were analyzed using zymography. The concentrated cultures were diluted with dH_2O and loading buffer (final concentration; 250 mM Tris-HCl pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS) and 0.015% bromophenol blue) to a final protein concentration of 1 μg/μl. Zymography was carried out with the method described in Koga et al. (2019a). Briefly,

the separating gel was prepared with gluten as a substrate (Amersham Life Science, Cleveland OH, US; final concentration, 2 mg/ml with 0.5% w/v SDS and 2.5 mM tris(2-carboxyethyl)phosphine hydrochloride). For all isolates and growth media combinations, 10 μg of secreted proteins were separated on a zymogram co-polymerized with gluten at 100 V for 4 h. In each zymogram, 8 μl Precision Plus Protein Dual Xtra Standard (Bio-Rad) was included. Stained zymograms were scanned with Epson Perfection 4990 Photo (EPSON, Nagano, Japan).

3. Results

3.1. Wheat quality parameters in samples with weak and strong gluten

Wheat grain samples were chosen for this study with the objective of having samples with either strong or weak gluten that met the Norwegian quality requirements for food-grade. The requirements for food-grade wheat are: falling number > 200 s, deoxynivalenol < 1250 μg/kg, and total protein of 10% (before 2014) or 11.5% (from 2014) (www.fk.no).

The grain samples had a falling number in the range 233 s to 433 s combined with an acceptable level of total protein, except sample A which had 9.2% protein (Table 1). Deoxynivalenol levels were moderate to low, except for two samples that were close to, or marginally exceeded, the 1250 μg/kg regulatory threshold (EC, 2006), i.e. sample C with 1128 μg/kg and sample F with 1391 μg/kg deoxynivalenol.

Falling number was slightly lower in samples with weak gluten (range of 233–404 s, mean of 302 s), compared to samples with strong gluten (range of 308–433 s, mean of 368 s) (Table S3). The total protein level was similar between the two groups, with a range of 9.2 to 13.3% (mean of 11.8%) in the samples with weak gluten, and a range of 10.7 to 12.4% (mean of 11.4%) in the samples with strong gluten. Concentrations of deoxynivalenol were higher in samples with weak gluten (range of 33–1391 μg/kg, mean of 466 μg/kg), compared to samples with strong gluten (range of 33–1291 μg/kg, mean of 119 μg/kg).

3.2. Microbial profiling by sequencing (metabarcoding)

The fungal communities across all grain samples had an average number of OTUs of 144 ± 21 (Table S4). There was no difference in the number of OTUs in samples with weak compared to strong gluten ($p = 0.571$ in model S1, Fig. S1), and no association between the number of OTUs and the total amount of fungal DNA ($p = 0.753$ in model S2). However, the wheat samples with strong gluten showed a larger variance in the number of OTUs than the samples with weak gluten ($p = 0.019$ in model S3). No association was observed between the community diversity and gluten strength ($p = 0.571$ in model S4, Fig. S1), or between the community diversity and the total amount of fungal DNA ($p = 0.943$ in model S5). The variance of community diversity did not differ between wheat grain with weak and strong gluten ($p = 0.166$ in model S6). Models S1-S6 are shown in Appendix 3.

The analysis of the distances between the microbial communities (β-diversity) in the samples resulted in four main clusters (Fig. 1), two for each type of wheat (spring, winter). For the spring wheat samples, two clusters linked to both location and gluten strength were revealed: Cluster 2 consisted of samples with weak gluten, all from Nes (Akershus) in 2012, and cluster 3 consisted of samples from Holmestrand (Vestfold) from 2012 and 2013 with all but one sample having strong gluten. Cluster 1 included three winter wheat samples, one sample each with weak and strong gluten from Ullensaker (Akershus) in 2014, and one with strong gluten from Stange (Hedmark) in 2012. Cluster 4 included seven samples of winter wheat of four different origins, that subclustered into four samples with weak and three samples with strong gluten.

Taxonomically, the most abundant OTUs detected in the metabarcoding belonged to plant pathogens, several saprophytes, and yeast (Table 3). Among the most abundant OTUs were those of four common wheat pathogens: OTU 1_*P. nodorum*, OTU 3_*M. majus*, OTU 4_*F. culmorum/graminearum*, and OTU 8_*F. avenaceum*, with relative abundancies across all samples in the range 0.01–0.94 (OTU 1), 0.01–0.6 (OTU 3), < 0.01–0.24 (OTU 4), and < 0.01–0.1 (OTU 8), respectively (Fig. 2A and Table S3). Less abundant OTUs belonging to the plant pathogens were OTU 6_*Neoasochyta* (0.01–0.22), OTU 10_*Pyrenophora* (< 0.01–0.07), OTU 20_*Pyrenophora* 2 (< 0.01–0.01), and OTU 19_*F. poae* (< 0.01–0.01). In addition to wheat pathogens, metabarcoding identified OTUs of well-known saprophytes in all samples: OTU 2_*Epicoccum* (0.01–0.39), OTU 5_*Cladosporium* (< 0.01–0.52), and OTU 7_*Alternaria* (< 0.01–0.2).

Mean relative abundancies of OTU 1_*P. nodorum* were lower in samples with weak gluten compared to those with strong gluten (0.22 vs. 0.43) (Fig. 3A and Table S3). Although much less pronounced, this was also observed for OTU 2_*Epicoccum* (0.18 vs. 0.22), OTU 7_*Alternaria* (0.03 vs. 0.05), and OTU 5_*Cladosporium* (0.07 vs. 0.09). Mean relative abundancies of OTU 3_ *M. majus* were higher in samples with weak compared to strong gluten (0.28 vs. 0.06), and slightly higher for OTU 4_*F. culmorum/graminearum* (0.09 vs.0.05), OTU 8_*F. avenaceum* (0.03 vs. 0.01), and OTU 6_*Neoacochyta* (0.06 vs. 0.03). Mean relative abundancies for all other OTUs were below 0.01 in wheat samples of both weak and strong gluten.

The sequences obtained in this study are available in the European Nucleotide Archive database under accession number PRJEB15346.

3.3. Quantification of DNA of selected fungal species (qPCR)

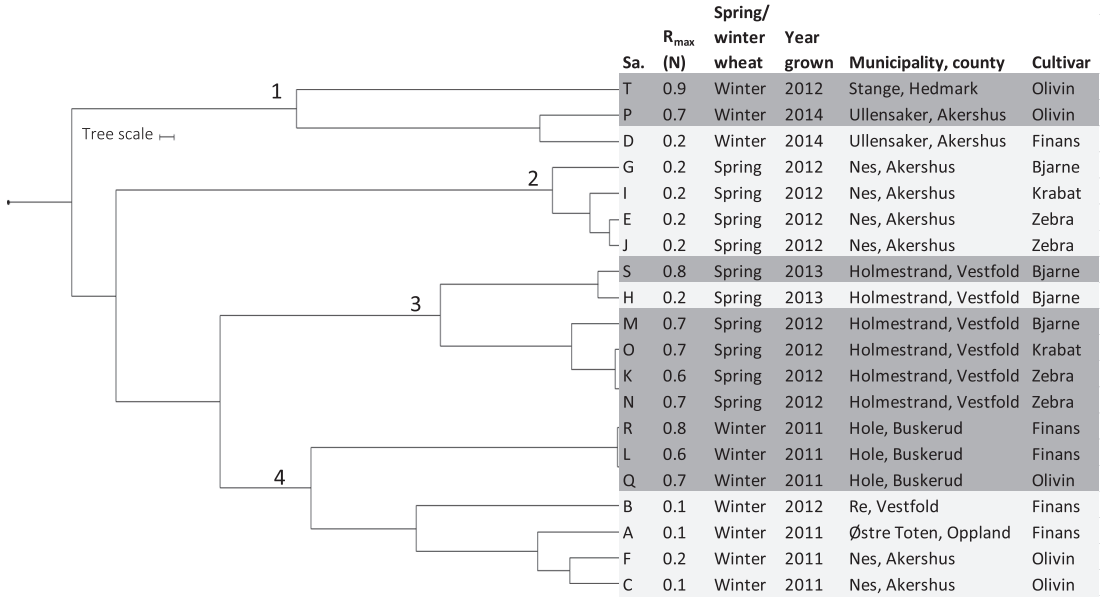
The total amount of fungal DNA estimated using the primers ITS1f and ITS2 ranged from 800 to 20,346 pg/μg (Fig. S2). Of the fungal species we quantified using species-specific qPCR, *M. majus* had the highest amounts of DNA with a range of 31–25,288 pg/μg (mean of 5055 pg/μg) across the 20 grain samples (Fig. 2B). *Microdochium majus* was followed by *P. nodorum* (range of 24–4732, mean of 1687 pg/μg), *F. graminearum* (range of 0–2432, mean of 620 pg/μg), *M. nivale* (range of 0–1929, mean of 401 pg/μg), and *F. avenaceum* (range of 0–538, mean of 88 pg/μg). DNA from *F. poae* and *F. culmorum* was scarcely detected at all, with mean levels of DNA across the 20 samples of 17 and 14 pg/ug, respectively. DNA from *F. sporotrichioides* was not detected.

The total amount of fungal DNA was higher in samples with weak gluten (range of 4769–20,346 pg/μg, mean of 10,592 pg/μg) than in the samples with strong gluten (range of 800–7299 pg/μg, mean of 3928 pg/μg). The mean level of DNA from *M. majus* (8963 vs. 1147 pg/μg), *M. nivale* (570 vs. 233 pg/μg), *F. graminearum* (972 vs. 268 pg/μg), and *F. avenaceum* (146 vs 30 pg/μg), were higher in samples with weak compared to strong gluten (Fig. 3B and Table S3). The mean level of DNA from *P. nodorum* was slightly lower in samples with weak compared to strong gluten (1498 vs. 1875 pg/μg). The other species measured by qPCR were present at low levels in samples with both weak and strong gluten.

3.4. Associations between gluten strength and fungal content

For the initial analyses of factors associated with gluten strength, falling number, total protein content, deoxynivalenol content, and total fungal DNA quantity were included in the GLM analyses. A negative association was detected between gluten strength (R_{max}) and total fungal DNA ($p < 0.05$, models S7 & 8, $R^2 > 70\%$). Stepwise analyses using the same covariates but replacing total fungal DNA with qPCR data for the five wheat pathogenic species, resulted in a model (model S9) with R^2 of 44% that included falling number ($F = 4.28$, $p = 0.054$) and *M. majus* DNA ($F = 3.55$, $p = 0.077$).

Stepwise regression was used to assess possible associations between gluten strength, and the continuous predictors wheat quality parameters (falling number, total protein, deoxynivalenol), DNA content of fungal species (qPCR), the total fungal DNA (qPCR), and the



Sa.	R_{max} (N)	Spring/ winter wheat	Year grown	Municipality, county	Cultivar
T	0.9	Winter	2012	Stange, Hedmark	Olivin
P	0.7	Winter	2014	Ullensaker, Akershus	Olivin
D	0.2	Winter	2014	Ullensaker, Akershus	Finans
G	0.2	Spring	2012	Nes, Akershus	Bjarne
I	0.2	Spring	2012	Nes, Akershus	Krabat
E	0.2	Spring	2012	Nes, Akershus	Zebra
J	0.2	Spring	2012	Nes, Akershus	Zebra
S	0.8	Spring	2013	Holmestrand, Vestfold	Bjarne
H	0.2	Spring	2013	Holmestrand, Vestfold	Bjarne
M	0.7	Spring	2012	Holmestrand, Vestfold	Bjarne
O	0.7	Spring	2012	Holmestrand, Vestfold	Krabat
K	0.6	Spring	2012	Holmestrand, Vestfold	Zebra
N	0.7	Spring	2012	Holmestrand, Vestfold	Zebra
R	0.8	Winter	2011	Hole, Buskerud	Finans
L	0.6	Winter	2011	Hole, Buskerud	Finans
Q	0.7	Winter	2011	Hole, Buskerud	Olivin
B	0.1	Winter	2012	Re, Vestfold	Finans
A	0.1	Winter	2011	Østre Toten, Oppland	Finans
F	0.2	Winter	2011	Nes, Akershus	Olivin
C	0.1	Winter	2011	Nes, Akershus	Olivin

Fig. 1. β-Diversity calculated as the distances between the microbial communities (Thetayc in Mothur v1.40.5) based on ITS1 microbial profiling among samples (Sa.) of spring or winter wheat with weak gluten ($R_{max} \leq 0.2$ N, grey) or strong gluten ($R_{max} \geq 0.6$ N, dark grey). Number 1–4 represent main clusters.

abundancy of (number of sequences) the 35 most common OTUs detected by metabarcoding, and the categorical predictors year and winter/spring wheat (or year and wheat variety). This analysis resulted in models with several predictors, including OTU 10_*Pyrenophora* detected by metabarcoding and *F. culmorum* detected by qPCR (not shown). Due to multicollinearity issues (VIF > 5), these two predictors were excluded from the final models.

The final model included six predictors and explained 97% of the variation in R_{max} (Eq. (1), and model S10). The predictor most strongly negatively associated with gluten strength was *F. avenaceum* measured by qPCR (coefficient of −0.18, F = 70.9, p = 0.000), followed by OTU 6_*Neoacochyta* (coefficient of −0.13, F = 46, p = 0.000), and OTU 3_*M. majus* (coefficient of −0.13, F = 39, p = 0.000). Additionally, a weak negative association was observed between OTU 32_*Alternaria* 3 and gluten strength (coefficient of −0.08, F = 13, p = 0.004). One predictor was significantly positively associated with gluten strength: OTU 7_*Alternaria* (coefficient of 0.14, F = 47, p = 0.000). Falling number was also included in the final model, though its positive association was not significant (coefficient of 0.05, F = 4.6, p = 0.053). The model was identical irrespective of the fixed factors used (year and winter/spring wheat, or year and wheat variety).

$$R_{max} = 0.471 - 0.001373\ F.\ avenaceum\ (\text{qPCR}) - 0.000003\ \text{OTU 3\_}M.\ majus - 0.000008\ \text{OTU 6\_}Neoascochyta - 0.000319\ \text{OTU 32\_}Alternaria\ 3 + 0.000008\ \text{OTU 7\_}Alternaria + 0.000901\ \text{falling number} \quad (1)$$

Models S7-S10, with OTU abundance used for the modeling, are shown in detail in Appendix 3.

3.5. Growth of selected fungi on different nitrogen sources

Three isolates each of *F. avenaceum*, *F. graminearum*, *M. majus*, and *M. nivale* all grew on the different media at 15 °C (Fig. S3, Appendix 2). Since some isolates had reached the margin of the Petri dishes by day seven, the average growth rates were calculated based on growth registrations from day three to six (Fig. 4). We used GLM to analyze the relationship between the average daily growth rates and the following factors: fungal species, growth medium, and their interaction. All three factors were significant (p = 0.000), and the model explained 79% of the variation in average daily growth rate (model S11, Appendix 3). The pairwise comparison of the average growth rates of all possible species and media combinations indicated that all *Fusarium* and *Microdochium* isolates and species tested grew equally well, or in case of *Microdochium*, even slightly faster on the gluten medium compared to all the other media we tested (model S12, Appendix 3).

3.6. Gluten-degrading ability of selected fungi (zymography)

To assess their gluten-degrading ability, one isolate each of *F. avenaceum*, *F. graminearum*, *M. majus*, *M. nivale*, and *P. nodorum* were grown in liquid cultures containing the complete medium or the minimal medium amended with gluten. The proteins secreted by the isolates were concentrated and separated on a zymogram gel copolymerized with gluten. White smears, or weak bands in the case of *F. avenaceum*, indicated the degradation of substrate (gluten proteins) by proteases for all the fungal isolates on the two growth media (Fig. 5). The appearance of smears rather than distinct bands in the zymogram indicated that some of the secreted fungal proteases remained active under the denaturing conditions with SDS and degraded the substrate during electrophoresis (similar to pronase as shown by Lantz and Ciborowski (1994)).

The longest smears were observed for proteins secreted by the isolate of *F. graminearum*, *P. nodorum* and the two *Microdochium* species, with variation in the length of the smears depending on the growth medium (Fig. 5). In the case of *F. graminearum*, the protein secreted by this isolate in the complete medium resulted in a smear that was more than double the length of the one generated from proteins secreted in the minimal medium. In case of *F. avenaceum*, pale bands were barely visible at the top of the separating gel. To investigate whether the proteins secreted by *F. avenaceum* had abilities to digest other types of substrates, they were also separated on a gelatin zymogram. In this zymogram, the protein secreted by *F. avenaceum* gave rise to clear smears (data not shown).

4. Discussion

If our samples were representative of grain lots at delivery, all would likely have met the food-grade quality requirements, including those that exhibited weak gluten. To identify fungi associated with reduced gluten

Table 3
Taxonomic assignment according to UNITE and NIBIO reference isolates, and applied taxonomic assignment, of the 35 most abundant operational taxonomic units (OTUs) in 20 wheat samples with either weak gluten ($R_{max} \leq 0.2$ N) or strong gluten ($R_{max} \geq 0.6$ N). Disease or other association in wheat is described for the 14 most abundant OTUs.

OTU ID	No. of reads	Taxonomic assignment (UNITE)	UNITE SH-accession, or ref. isolate sequence[a]	Length (bp)	Coverage/identity[b]	Phylum	Class	Order	Family	Genus	Applied taxonomic assignment[c]	Disease or other association in wheat[d]
OTU 1	1817790	*Parastagonospora nodorum*	NIBIO 201 254	227	100/100	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	*Parastagonospora*	*Parastagonospora nodorum*	leaf and glume blotch
OTU 2	1133361	*Epicoccum nigrum*	SH117088.07FU	184	89/100	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Epicoccum*	*Epicoccum*	Saprophyte, black head mold, red blotch of grains
OTU 3	950858	*Microdochium majus*	NIBIO 200 349	186	100/100	Ascomycota	Sordariomycetes	Xylariales	Hyponecteriaceae	*Microdochium*	*Microdochium majus*	Snow mold, FHB, seedling blight[e]
OTU 5	451982	*Mycosphaerella tassiana*	SH216250.07FU	196	98/100	Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	*Mycosphaerella*	*Cladosporium*	Saprophyte, black head mold, black point[g,h]
OTU 4	381097	*Fusarium culmorum*	NIBIO 200 630 and 201 064	189	100/100	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*	*Fusarium culmorum/graminearum*	FHB, foot and root rot, seedling blight
OTU 6	271073	*Neoascochyta graminicola*	SH293210.07FU	182	89/100	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Neoascochyta*	*Neoascochyta*	Ascochyta leaf scorch/spot[f]
OTU 7	219593	*Alternaria infectoria*	SH434793.07FU	235	93/100	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Alternaria*	*Alternaria*	Saprophyte, black head mold, black point[g,h]
OTU 8	114678	*Fusarium avenaceum*	NIBIO 201 081	192	100/100	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*	*Fusarium avenaceum*	FHB, foot rot, seedling blight
OTU 10	44594	*Pyrenophora tritici-repentis*	SH193139.07FU	219	90/100	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Pyrenophora*	*Pyrenophora*	Tan spot (DTR), yellow leaf spot/blotch
OTU 14	32521	*Cladosporium delicatulum*	SH217942.07FU	197	92/100	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*	*Cladosporium*2	
OTU 16	25595	Pleosporales sp	SH174245.07FU	206	87/100	Ascomycota	Dothideomycetes	Pleosporales			*Alternaria* 2 (BOLD)	
OTU 18	25487	*Sporobolomyces phaffii*	SH259909.07FU	188	96/98.9	Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	*Sporobolomyces*	*Sporobolomyces*	Red/pink/white kernel discoloration[g]
OTU 15	23722	Fungi sp	SH526155.07FU	227	98/96.4	Ascomycota					*Phaeosphaeria* (BOLD)	
OTU 11	17099	*Sclerotinia borealis*	NIBIO 201 055	188	100/100	Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	*Sclerotinia*	*Sclerotinia*	Snow mold
OTU 20	7932	*Pyrenophora teres*	SH193140.07FU	220	87/99.5	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Pyrenophora*	*Pyrenophora* 2	
OTU 19	7903	*Fusarium poae*	NIBIO 200 871	180	100/100	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*	*Fusarium poae*	
OTU 24	6514	*Sporobolomyces roseus*	SH194973.07FU	193	86/100	Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	*Sporobolomyces*	*Sporobolomyces*2	

(*continued on next page*)

Table 3 (*continued*)

OTU ID	No. of reads	Taxonomic assignment (UNITE)	UNITE SH-accession, or ref. isolate sequence[a]	Length (bp)	Coverage/identity[b]	Phylum	Class	Order	Family	Genus	Applied taxonomic assignment[c]	Disease or other association in wheat[d]
OTU 23	5413	*Neoascochyta graminicola*	SH293210.07FU	183	89/95.1	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Neoascochyta*	*Neoascochyta* 2	
OTU 29	3932	*Epicoccum nigrum*	SH117088.07FU	183	89/95.1	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Epicoccum*	*Epicoccum* 2	
OTU 40	3183	Didymellaceae sp	SH215820.07FU	182	85/100	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Unidentified	Didymellaceae	
OTU 32	3043	*Alternaria rosae*	SH294936.07FU	238	89/100	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Alternaria*	*Alternaria* 3	
OTU 35	2604	Sordariomycetes sp	SH213518.07FU	182	85/100	Ascomycota	Sordariomycetes	Unidentified	Unidentified	Unidentified	*Pseudomicrodochium* (BOLD)	
OTU 51	2332	*Neoascochyta desmazieri*	SH215819.07FU	181	89/100	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Neoascochyta*	*Neoascochyta* 3	
OTU 37	2054	*Stagonospora pseudovitensis*	SH182977.07FU	234	88/98.6	Ascomycota	Dothideomycetes	Pleosporales	Massarinacea	*Stagonospora*	*Stagonospora*	
OTU 41	1978	*Didymella arachidicola*	SH345871.07FU	180	89/100	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Didymella*	*Didymella*	
OTU 49	1935	*Dioszegia hungarica*	SH182099.07FU	155	83/100	Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Dioszegia*	*Dioszegia*	
OTU 46	1905	*Dioszegia fristingensis*	SH209710.07FU	156	90/100	Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Dioszegia*	*Dioszegia* 2	
OTU 38	1749	*Bipolaris eleusines*	SH292483.07FU	212	91/99	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Bipolaris*	*Bipolaris*	
OTU 59	1322	*Itersonilia pannonica*	SH199073.07FU	195	86/100	Basidiomycota	Tremellomycetes	Cystofilobasidiales	Mrakiaceae	*Itersonilia*	*Itersonilia*	
OTU 58	1259	*Bullera alba*	SH215453.07FU	159	83/100	Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Bullera*	*Bullera*	
OTU 63	1208	*Vishniacozyma victoriae*	SH181628.07FU	175	85/100	Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*	*Vishniacozyma*	
OTU 52	1124	Phaeosphaeriaceae sp	SH183016.07FU	233	90/96.7	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Phaeosphaeriaceae	Phaeosphaeriaceae	
OTU 54	1110	Phaeosphaeriaceae sp	SH183031.07FU	233	91/99.1	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Phaeosphaeriaceae	Phaeosphaeriaceae 2	
OTU 70	1029	*Neoascochyta paspali*	SH215817.07FU	181	89/98.8	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Neoascochyta	*Neoascochyta* 4	
OTU 73	1009	*Vishniacozyma victoriae*	SH181632.07FU	175	85/100	Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	Vishniacozyma	*Vishniacozyma*	

[a] NIBIO reference isolates are shown in Table S1 (Appendix 1).

[b] Coverage/identity = Percent aligned sequence/Percent identical bases within aligned sequence.

[c] Taxonomic classification according to BOLD Identification System for ITS was used for OTUs that had poor taxonomic resolution according to UNITE fungal ITS (sh_general_release_dynamic_01.07.2017).

[d] According to the EPPO Global Database (https://gd.eppo.int), if not stated otherwise.

[e] Dill-Macky R (2010) Fusarium Head Blight. In: Bockus W, Bowden R, Hunger R, Morrill W, Murray T, Smiley R (eds) Compendium of Wheat Diseases and Pests. 3rd edition. The American Phytopathological Society, St.Paul, Minnesota, USA.

[f] Krupinsky JM, Cline E (2010) Ascochyta Leaf Spot. In: Bockus W, Bowden R, Hunger R, Morrill W, Murray T, Smiley R (eds) Compendium of Wheat Diseases and Pests. 3rd edition. American Phytopathological Society (APS Press), St.Paul, Minnesota, USA.

[g] Jacobsen B (2010) Black Head Molds. In: Bockus W, Bowden R, Hunger R, Morrill W, Murray T, Smiley R (eds) Compendium of Wheat Diseases and Pests. 3rd edition. The American Phytopathological Society, St.Paul, Minnesota, USA.

[h] Fernandez M (2010) Black Point. In: Bockus W, Bowden R, Hunger R, Morrill W, Murray T, Smiley R (eds) Compendium of Wheat Diseases and Pests. 3rd edition. The American Phytopathological Society, St.Paul, Minnesota, USA.

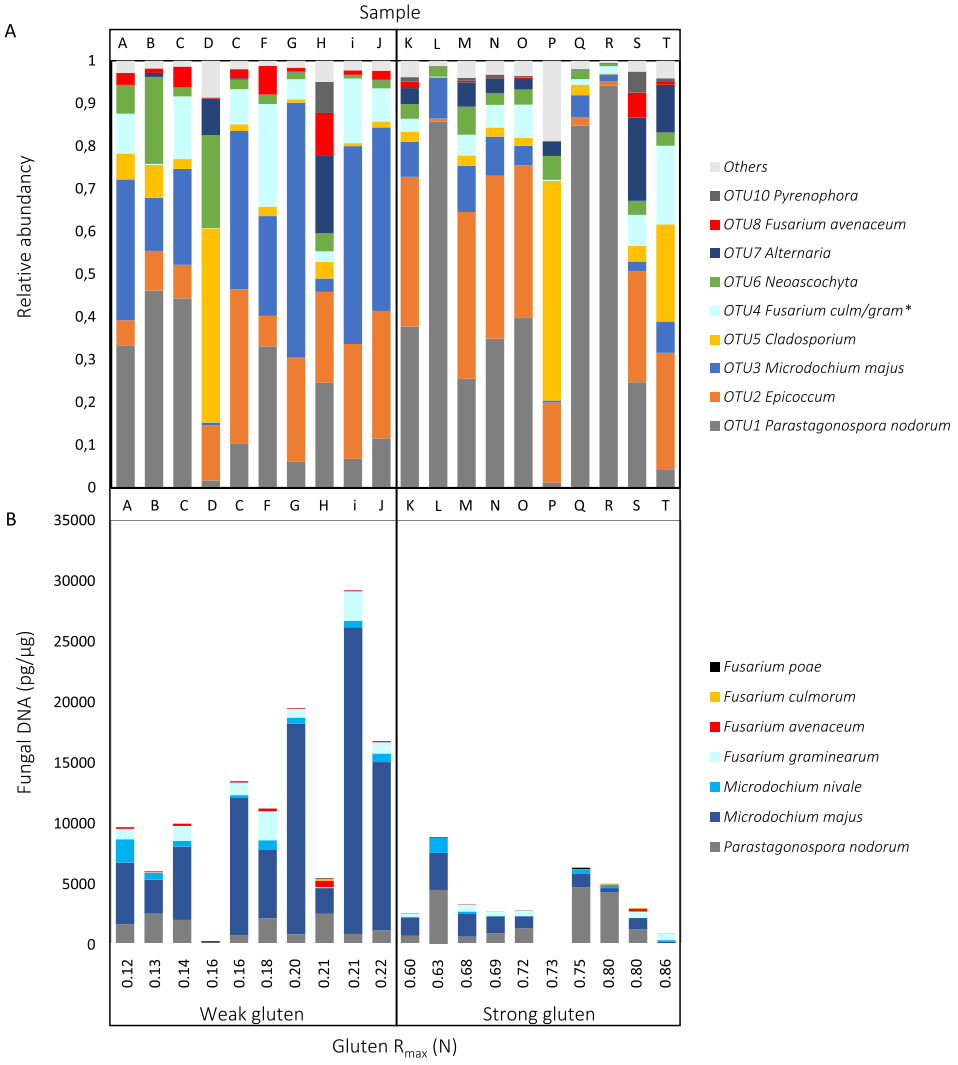


Fig. 2. The relative abundance of the nine most abundant operational taxonomic units (OTUs) identified with metabarcoding of Earthmicrobiome ITS1 (A), and the amount of DNA from a selection of fungal species quantified by qPCR (B), in 20 wheat samples with either weak gluten ($R_{max} \leq 0.2$ N, left panels) or strong gluten ($R_{max} \geq 0.6$ N, right panels). **Fusarium culmorum* or *Fusarium graminearum* (the species could not be separated by metabarcoding).

quality in Norwegian wheat grain, we used metabarcoding to compare fungal communities of wheat grain with weak and strong gluten. DNA content of common fungal wheat pathogens in the grain were also quantified using species-specific qPCR. In vitro experiments with gluten-amended media and zymography were used to confirm the ability of a selection of fungi to utilize gluten for their growth and to secrete gluten-degrading proteases. To our knowledge, this is the first study that uses metabarcoding to identify possible associations between the fungal communities and gluten quality in wheat grain.

4.1. Occurrence of fungi

We detected the following fungal species in relatively high amounts using metabarcoding: *P. nodorum* (OTU 1), *M. majus* (OTU 3), *F. culmorum* and/or *F. graminearum* (OTU 4), and *F. avenaceum* (OTU 8). Likewise, species-specific qPCR showed that of the species examined, *M. majus* had the highest average levels of DNA, followed by *P. nodorum*, *F. graminearum*, *M. nivale* and *F. avenaceum*.

The fungal pathogens detected in our study were consistent with results from earlier studies of fungal diseases in Norwegian cereals, in which the most common diseases observed in wheat grain were FHB, caused by *Microdochium* and *Fusarium* spp. (Hofgaard et al., 2016a; Hofgaard et al., 2009), and glume blotch caused by *P. nodorum* (Anonymous, 1975-2018). The FHB and glume blotch pathogens in Norwegian seed lots have been recorded every year since seed health analyses were started in 1974 (Anonymous, 1975-2018). Annual average seed infection levels since then have varied between 1 and 47% infected seeds for FHB pathogens, and 2 and 22% infected seeds for *P. nodorum*.

In addition to the common wheat pathogens, metabarcoding also revealed a number of other cereal pathogens including *Neoascochyta*, *Bipolaris*, and *Pyrenophora*. Opportunists or saprophytes including *Cladosporium*, *Alternaria*, and *Epicoccum*, as well as yeasts including *Sporobolomyces*, *Dioszegia*, *Vishniacozyma*, and *Itersonilia* were also detected. Nearly all of these fungi are known to be associated with wheat grain (Machacek et al., 1951; Nicolaisen et al., 2014; Ylimaki, 1981).

Metabarcoding could not separate the deoxynivalenol producing fusaria *F. graminearum* and *F. culmorum*, however, qPCR demonstrated that *F. graminearum* was the dominant deoxynivalenol producer in the wheat samples as DNA from *F. culmorum* was not detected, or at very low levels only.

4.2. Association between gluten strength and fungal content

The initial modeling indicated a negative association between fungal infection, measured as total fungal DNA by qPCR, and gluten

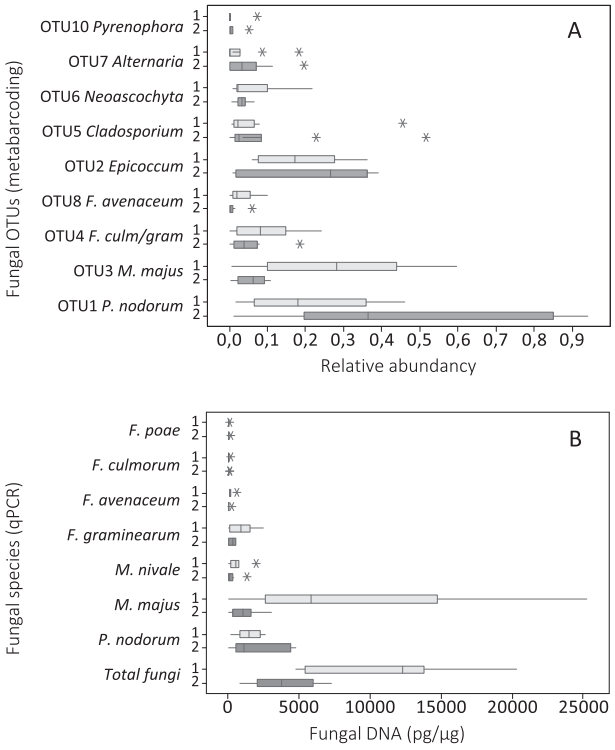


Fig. 3. The distribution of the relative abundancy of the most common fungal OTUs identified with microbial profiling by Earthmicrobiome ITS1 (A) or the amount of fungal DNA (pg/µg plant DNA) analyzed by qPCR (B) in 10 samples of wheat grain of weak gluten ($R_{max} \leq 0.2$ N, grey bars), and 10 of strong gluten ($R_{max} \geq 0.6$ N, dark bars). Each box shows median (black line), quartile 1 and 3 (bottom and top of box, respectively), and outliers (stars) of the relative OTU abundancy or fungal DNA. Total fungal DNA was assessed by qPCR in 19 samples.

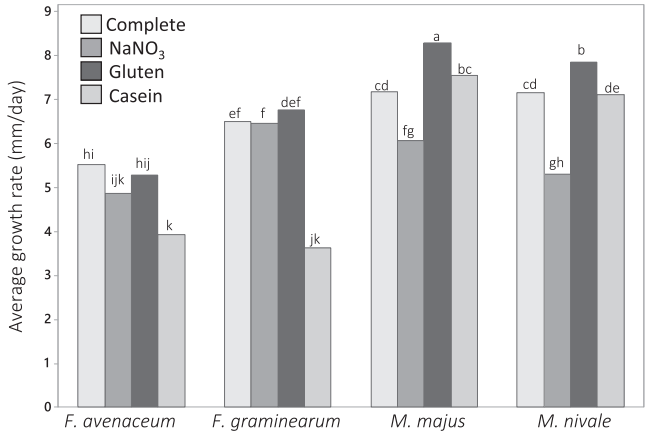


Fig. 4. Average mycelial growth rates (mm/day) of *Fusarium avenaceum*, *Fusarium graminearum*, *Microdochium majus*, and *Microdochium nivale* at 15 °C on agar containing various nitrogen sources. The media included were a complete medium (with yeast extract, $NaNO_3$, and casein); and three minimal media each with one of nitrogen source ($NaNO_3$, gluten, or casein). The growth rates were calculated as the average across three isolates per fungal species. Letters above the columns correspond to groups by Fisher LSD Method, 95% Confidence. Bars that do not share letters are statistically different.

strength in our samples. We detected four FHB pathogens that were present at higher average DNA levels in the samples with weak gluten than in the samples with strong gluten, i.e. *M. majus, F. graminearum, M. nivale*, and *F. avenaceum*. Since isolates of these four pathogen species also showed some ability for gluten degradation in vitro, it is possible

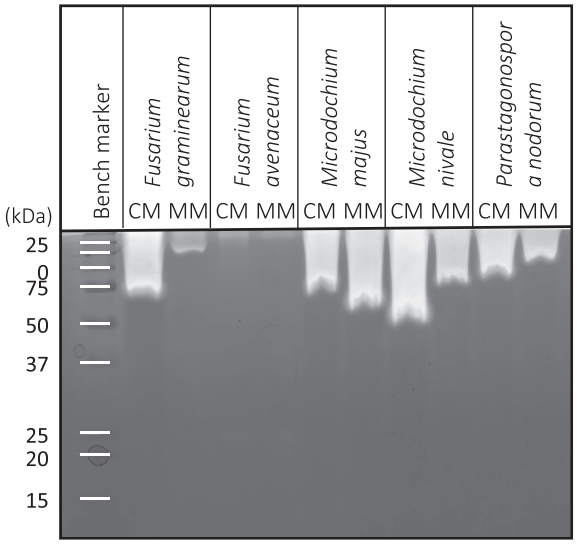


Fig. 5. Zymogram gels copolymerized with gluten proteins as a protein substrate. One isolate of each of five fungi were grown in a complete medium (CM; containing yeast extract, NaNO3 and casein) or a minimal medium (MM; with gluten as a nitrogen source) for seven days at 18 °C. Proteins secreted by fungi in each medium were separated on the gels. White smears indicate a degradation of gluten proteins. Bench marker: Precision Plus Protein Dual Xtra (BioRad).

that these species could have contributed to reducing gluten strength. In the more detailed stepwise regression analysis we attempted to identify which of the fungi that were most strongly associated with reduced gluten strength in our samples. This analysis revealed a negative association between gluten strength and the following predictors: *F. avenaceum* DNA content measured by qPCR, and abundance of OTU 3_*M. majus*, OTU 6_*Neoascohyta*, and OTU 32_*Alternaria* 3 from metabarcoding. OTU 7_*Alternaria* appeared positively correlated to gluten strength.

Of the fungal species that were negatively associated with gluten strength, *M. majus* stood out. Relatively high levels of *M. majus* DNA were observed by both metabarcoding and qPCR in connection with samples with weak gluten. *Microdochium nivale* was also detected at higher levels in samples of weak compared to strong gluten, though the DNA levels were lower than for *M. majus*. *Microdochium* spp. are common in wheat grain (Hofgaard et al., 2009; Ioos et al., 2004; Nielsen et al., 2013). However, the effect of *Microdochium* spp. on gluten or baking quality has yet to be elucidated. Blandino and Reyneri (2009) reported an increase in dough strength when flour was made from grain harvested from winter wheat treated with a combination of the fungicides azoxystrobin and prochloraz, compared to a treatment with prochloraz alone. This finding suggests a negative impact of *Microdochium* on dough strength, as azoxystrobin has an effect towards *Microdochium*, while prochloraz reduces both *Fusarium* and *Microdochium* (Matušinsky et al., 2017; Pirgozliev et al., 2003). In our growth rate study, isolates of both *Microdochium* species grew faster on the medium amended with gluten compared to the other media, implying that these fungal species have ample ability to utilize gluten for their growth. Moreover, with zymography, we observed that the proteins secreted by *M. majus* and *M. nivale* in liquid cultures were able to hydrolyze gluten. Among the proteins secreted, the ones that originated from *Microdochium* spp. resulted in the longest smears in the zymogram. Lantz and Ciborowski (1994) reported that when proteases are active during electrophoresis, the length of a smear in a zymogram increases with the amount of proteases in the sample. This suggests that the proteins secreted by *Microdochium* had more gluten degrading proteases than the other fungi we tested in our study. These results support the hypothesis that *Microdochium,* like some *Fusarium* species, can secrete

proteases that digest gluten and thereby contributing to reducing the baking quality of wheat.

Fusarium avenaceum, as measured by qPCR, was also negatively associated to gluten strength in our regression modeling. This fungus has been associated with reduced baking quality of naturally infected wheat (Nightingale et al., 1999) and altered gluten protein composition and reduced gluten strength in artificially inoculated flour (Bellesi et al., 2019). In our growth rate tests, the three isolates of *F. avenaceum* were all able to utilize gluten, growing at comparable rates on the gluten medium and the complete medium. Zymography showed that proteins secreted by *F. avenaceum* digested gluten to a low degree, and a gelatin zymogram (not shown) confirmed that this species secreted proteases that degraded gelatin. Since we observed that the DNA quantities of this fungus were relatively low compared to other fungi in wheat samples with weak gluten, we speculate that the strong negative association of *F. avenaceum* with gluten strength may have resulted from a covariate of *F. avenaceum*, not only the fungus itself. Based on the slightly confusing line of evidence, further study of the effect of *F. avenaceum* on wheat gluten and baking quality is warranted.

The mean level of *F. graminearum* was higher in samples of weak compared to strong gluten, and the level exceeded that of *F. avenaceum*. Unlike *F. avenaceum*, *F. graminearum* was not associated with gluten strength in our regression modeling. Examining the DNA levels in each sample revealed that *F. graminearum* was present in most of the samples with strong gluten, and the levels were only slightly lower than in most of the samples of weak gluten (results not shown). *Fusarium avenaceum* on the other hand, was present in all but one sample of weak gluten and was not present (except for one instance) in the samples of strong gluten (results not shown). This could explain why the association to gluten strength was less clear for *F. graminearum* than for *F. avenaceum*, particularly since the modeling included standardization of the predictors to even out scale differences. *Fusarium graminearum* is the main species causing FHB in cereals world-wide and is recognized as the main producer of deoxynivalenol in Norwegian cereals (Hofgaard et al., 2016a). Studies have linked *F. graminearum* to reduced baking quality (Dexter et al., 1996; Koga et al., 2019a; Koga et al., 2019b; Nightingale et al., 1999). The reduction in baking quality associated with *F. graminearum* is suggested to be caused by reduced levels of the larger glutenin polymers and/or the presence of gluten degrading proteases in *Fusarium*-infested grain. The three Norwegian *F. graminearum* isolates we analyzed in our growth rate test utilized gluten for their growth. The zymography test similarly confirmed its ability to secrete gluten-degrading proteases. Compared to the survey of *Fusarium* and mycotoxins in Norwegian cereals in 2004–2009 (Hofgaard et al., 2016a), the levels of *F. graminearum* and deoxynivalenol in our present study were moderate to low, with only two samples containing deoxynivalenol levels near the limit acceptable for food-grade wheat. This could suggest that the *F. graminearum* levels in our present study were too low to be strongly associated with gluten strength. In Norway, deoxynivalenol is tested upon wheat grain delivery, and grain lots that are heavily contaminated with deoxynivalenol producing fungi are unlikely to be classified as food-grade.

Another predictor that was negatively associated with gluten strength in our modeling analyses was OTU 6_*Neoascohyta*. OTU 6_*Neoascochyta* was detected in all samples, though in low abundancies. Chen et al. (2015) revised the taxonomy of the *Didymellaceae* family and thus some of the previously reported *Ascochyta* species are now classified as *Neoascochyta*. *Neoascochyta* is considered a weak pathogen causing Ascochyta leaf spot on wheat worldwide (Krupinsky and Cline, 2010). *Neoascochyta exitilais* (syn. *Didymella exitialis*) was one of the most abundant fungus in Danish and Swedish studies of fungal communities in wheat grain (Grudzinska-Sterno et al., 2016; Hertz et al., 2016; Nicolaisen et al., 2014). In Norway, *Ascochyta* spp. have been reported on wheat seeds (Overaa, 1978). To our knowledge, the relationship between this fungus and gluten quality in wheat has not previously been examined.

OTU 7_*Alternaria* was the only predictor that was positively associated with gluten strength. The association appeared as moderate to strong, and this OTU was observed in moderate to high abundancies in most of the samples with strong gluten, and less so in samples with weak gluten. These results are consistent with the findings of Nightingale et al. (1999) who observed a high rate of *Alternaria alternata* in kernels that yielded flour of stable dough quality. The positive association between *Alternaria* and gluten or dough quality could be linked to the antagonistic activity of *Alternaria* spp. towards fungal pathogens such as *Microdochium*, as observed by Bateman (1979). It should be mentioned that we observed a negative but weak association between another *Alternaria* OTU (OTU 32) and gluten strength. This OTU was mainly detected in one sample (D), being otherwise detected at low relative abundancies. *Alternaria* (syn. *Lewia*) are among the most abundant fungi on cereal grains (Grudzinska-Sterno et al., 2016; Hertz et al., 2016; Kosiak et al., 2004; Nicolaisen et al., 2014; Overaa, 1978). *Alternaria* may cause the disease black point of wheat, which is visible as a darkening in the embryo end of the grain (Culshaw et al., 1988; Perello et al., 2008). Black point of wheat has been associated with reductions in a number of quality measures including dough stability (Rees et al., 1984), dough strength (Goswami and Sehgal, 1969), and bread volume (Lorenz, 1986). *Alternaria* species are morphologically similar, and there has been taxonomical confusion within the genus (Andersen et al., 1996). Our study indicated a contrasting effect of different *Alternaria* OTUs on gluten strength. We speculate whether different *Alternaria* species, or different levels of infection, could be involved. The abundance and effect of different *Alternaria* species on wheat quality remains to be investigated.

Another fungus that we observed in a relatively high amount with both qPCR and metabarcoding was *P. nodorum*, which can cause leaf and glume blotch of wheat. In contrast to *Microdochium* and *Fusarium* spp., the DNA of *P. nodorum* tended to be present at higher levels in the samples with strong gluten than in those with weak gluten, though it was not associated with gluten strength in our regression models. Karjalainen and Salovaara (1988) observed that grain from wheat inoculated with *P. nodorum* was associated with increased protein content and better rheological properties of the dough and test baking results, compared to grain from un-inoculated plants. The positive effects on grain quality were explained by severe yield reductions due to reduced photosynthetic assimilation (starch synthesis) in infected leaves, which increased the grain protein content. The study did not include data on disease development or indicate whether the fungus was present in grain. In our study, the proteases secreted by this species exhibited a high degree of proteolytic breakdown of gluten, suggesting that like *Microdochium* and *Fusarium*, this fungus has the potential to degrade gluten. However, since *P. nodorum* was present at relatively high levels in our grain with strong gluten, we find it less likely that this fungus was contributing to reduced gluten strength in our materials.

In our study, metabarcoding also revealed the presence of two common saprophytes, *Epicoccum* (OTU 2) and *Cladosporium* (OTU 5), that did not appear to be associated with gluten strength. *Epicoccum nigrum* (*syn E. purpurascens*), a common saprophyte on seeds and other plant materials, is reported from many studies of fungi on wheat seed (Lević et al., 2012; Nicolaisen et al., 2014; Overaa, 1978; Ylimaki, 1981) and was one of the most abundant species in our study. This fungus has been investigated for antagonistic activity against fungal pathogens, including *F. graminearum* (Jensen et al., 2016; Ogórek and Plaskowska, 2011). In our material, *Epicoccum* (OTU 2) was detected in all samples regardless of gluten strength. *Cladosporium* (OTU 5) was present at low abundances in all but two samples from Romerike in 2014, one of which had strong gluten and the other weak. *Cladosporium* spp. are ubiquitous and found worldwide on a number of substrates, including living, senescing and dead plant materials. *Cladosporium* spp. are frequent saprophytes on seeds of several plant species, including wheat (Grudzinska-Sterno et al., 2016; Hertz et al., 2016; Lević et al., 2012; Machacek et al., 1951; Nicolaisen et al., 2014; Ylimaki, 1981).

Among the OTUs belonging to pathogens that were less common in our material, was OTU 10_*Pyrenophora tritici-repentis*, the common agent of tan spot (DTR) in wheat, though the relative abundancies of the OTU were generally low. *Pyrenophora tritici-repentis* has been detected sporadically on Norwegian seeds during the years and the disease has been observed occasionally in wheat fields (Brodal, unpublished). The OTU was not included in the final regression model, suggesting that it was not associated with gluten strength in our material.

4.3. Associations between gluten strength and other factors (cultivar, weather)

Two of the samples in our study, sample D and P, had microbial profiles that were very different from the other samples examined. Their fungal profiles being dominated by OTU 5_*Cladosporium* and OTU 6_ *Neoascohyta*, whereas levels of *P. nodorum*, *Fusarium* and *Microdochium* species were low or absent. Both samples were collected in 2014 from the same field in Romerike but were from different cultivars, Finans and Olivin. FHB pathogens generally rely upon a period of high humidity to establish infection. The summer of 2014 in Norway was exceptionally hot and dry (www.met.no/publikasjoner/met-info/met-info-2014). The prevailing weather likely explains the absence of the FHB pathogens in our 2014 samples. Despite the fact that these two samples originated from the same field and had similar fungal profiles that set them apart from the other samples, they differed largely in gluten quality: Finans (D) showed an almost complete loss of gluten strength, whereas Olivin (P) had strong gluten. Therefore, we suspect that the large difference in gluten quality could likely be attributed to other factors, such as gluten-degrading proteases produced by organisms other than fungi. The interaction between wheat genotype and environment could also have played a role in the observed differences in gluten quality. In annual quality assessment trials, Finans has been predisposed to environmental influence, resulting in considerable variation in gluten quality.

All the grain samples in our study had falling number within food grade (> 200 s). Despite this, the grain samples with weak gluten had a lower average falling number, as well as a higher level of fungal DNA, than those with strong gluten. The falling number indirectly measures the activity of the starch-degrading enzyme, α-amylase, and is used to detect sprout damaged grain (Hagberg, 1960). In addition to the activation of cereal α-amylase upon sprouting, many fungi can secrete amylases, and instances of *Fusarium* damaged grain with increased activity of α-amylase have been reported (Dexter et al., 1996; Wang et al., 2008). Humid conditions after grain maturation and before harvest triggers pre-harvest sprouting and the synthesis of α-amylase in the grain as well as promoting fungal growth and development. The relationship between fungal α-amylase and falling number is not clear, as increased levels of fungal α-amylase are not necessarily associated with reduced falling number (Wang et al., 2008). Based on existing knowledge, we cannot conclude whether the reduced falling number in our grain samples with weak gluten was a result of fungal infection or the initiation of the pre-harvest sprouting process in the grain.

In our final stepwise model, both *F. avenaceum* and *Microdochium* were negatively associated with gluten strength. These are fungi that have been associated with relatively cool and humid conditions (Parry et al., 1995; Xu et al., 2008), as reflected by their optimum temperatures for in vitro growth of 15–20 °C, which is notably lower than those of 20–25 °C for *F. graminearum*, *F. culmorum* and *F. poae*. Weakening of gluten in field-grown wheat has been associated with diurnal temperatures below 18 °C during heading and grain filling (Moldestad et al., 2011). However, little negative effect of low temperature on gluten quality has been reported in grain grown under controlled conditions (Koga et al., 2015, 2016a). Moreover, Uhlen et al. (2015) observed an inconsistent relationship between temperature and gluten strength in field-grown wheat and suggested that the weakening of the gluten was caused by factors related to the low temperature conditions. During summer, the temperature tends to drop during periods with rainfall. These are conditions that are likely to favor the development of fungi, particularly those that thrive at lower temperatures such as *F. avenaceum* and *Microdochium*. In addition to having the ability to grow under cooler conditions, these fungi rely upon a period of high humidity to establish infection during periods of host plant susceptibility, from anthesis to soft dough (Andersen, 1948). In light of our findings and those of others, further study of the effect of *F. avenaceum,* and particularly *Microdochium* species, on gluten quality in wheat is merited.

5. Conclusion

In Norway, the variation in gluten quality caused by environmental factors is not accounted for at grain delivery. Grain lots with weak gluten can pass unnoticed into the food or bread-making grade contributing to instabilities in gluten strength. We detected four FHB pathogens that were present at higher levels in grain samples with weak gluten compared to those with strong gluten. Isolates of these fungal species were able to utilize and degrade gluten in vitro, and it is possible that all four FHB pathogens contributed to reduced gluten strength. A more detailed stepwise regression analysis revealed a negative association between gluten strength and the DNA levels or abundance of certain fungi. *Microdochium majus* was the species that dominated in samples with weak gluten. Despite being present at relatively low levels, *F. avenaceum* also appeared to be negatively associated to gluten strength. To minimize problems with instabilities in gluten strength in food-grade wheat, further investigations into the role of different fungal species with respect to gluten strength are required.

Author contributions

Heidi Udnes Aamot: Conceptualization, Methodology, Investigation, Formal analysis, Writing - Original Draft. Erik Lysøe: Methodology, Formal analysis, Writing - Review & Editing. Shiori Koga: Conceptualization, Supervision, Resources, Visualization, Methodology, Investigation, Writing - Review & Editing. Katherine Ann Gredvig Nielsen: Investigation, Formal analysis, Writing - Review & Editing. Ulrike Böcker: Resources, Writing - Review & Editing. Guro Brodal: Supervision, Writing - Review & Editing. Ruth Dill-Macky: Writing - Review & Editing. Anne Kjersti Uhlen: Conceptualization, Resources, Writing - Review & Editing, Project administration, Funding acquisition. Ingerd Skow Hofgaard: Conceptualization, Project administration, Supervision, Writing - Review & Editing, Resources.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2020.108712.

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