Breeding selection imposed a differential selective pressure on the wheat root-associated microbiome

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Abstract

Plants-microbiome associations are the result of millions of years of co-evolution. Due to breeding-accelerated plant evolution in non-native and highly managed soil, plant-microbe links could have been lost. We hypothesized that post-domestication breeding of wheat changed the root-associated microbiome. To test this, we analyzed root-associated fungal and bacterial communities shortly after emergence of seedlings representing a transect of wheat evolution including modern wheat, landraces, and ancestors. Numbers of observed microbial taxa were highest in landraces bred in low-input agricultural systems, and lowest in ancestors that had evolved in native soils. The microbial communities of modern cultivars were different from those of landraces and ancestors. Old wheat accessions enriched *Acidobacteria* and *Actinobacteria*, while modern cultivars enriched OTUs from *Candidatus Saccharibacteria*, *Verrucomicrobia* and *Firmicutes*. The fungal pathogens *Fusarium*, *Neoascochyta* and *Microdochium* enriched in modern cultivars. Both bacterial and fungal communities followed a neutral assembly model when bulk soil was considered as the source community, but accessions of the ancient *Triticum turgidum* and *T. monococcum* created a more isolated environment in their roots. In conclusion, wheat root-associated microbiomes have dramatically changed through a transect of breeding history.

Keywords: Breeding, Microbiome, Root, Wheat, Bacteria, Fungi, Selective pressure.

Background

Plants are colonized by massive diversity of microorganisms and the rhizosphere is considered one of the most active microbiological environments on Earth (Berendsen *et al.*, 2012). These plant-microbiome associations are the result of millions of years of co-evolution (Baltrus, 2017), and it is well documented that host genotype has significant, albeit minor, effects on microbial community composition, both aboveground (Sapkota *et al.*, 2015) and belowground (Lundberg *et al.*, 2012, Peiffer *et al.*, 2013). Many plant-associated microorganisms are crucial for plant growth, nutrient acquisition, as well as protection against biotic and abiotic stresses (Finkel *et al.*, 2017). In contrast to natural evolution, domestication is a fast anthropological selection of crops, which, like natural selection, is based on genetic diversity and selection of desired traits. Modern breeding has further accelerated evolutionary processes with a focus on selection of fertilizer-responsive high-yielding and disease-resistant cultivars. In parallel, agriculture has undergone dramatic intensification during the last century with increasing agronomic management including higher levels of fertilizer and pesticide inputs. Considering breeding is a result of human-induced selection rather than natural evolution, our understanding of breeding-induced evolutionary

interactions between microbiota and plant traits is only emerging (Perez-Jaramillo *et al.*, 2018, Escudero-Martinez & Bulgarelli, 2019).

It is evident that modern wheat is a result of changes of the plant genome through hybridization events and modification of genes in the process of selection of desirable plant traits. Such selection pressure for high yield in combination with high-input agriculture and neglect of root processes and performance, may inadvertently have led to depletion of members of the root microbiota that were important for nutrient acquisition and pathogen control in natural low-input conditions (Perez-Jaramillo *et al.*, 2016, Kavamura *et al.*, 2020, Tkacz *et al.*, 2020). In common sunflower (*Helianthus annuus*) domestication affected fungal communities, but not bacterial communities, and modern sunflower varieties had a lower relative abundance of putative fungal pathogens (Leff *et al.*, 2017). Similarly, bacterial isolates from sugar beet roots (*Beta vulgaris* ssp. *vulgaris*) were more active against phytopathogens, compared to isolates from the wild ancestor (*B. vulgaris* ssp. *maritima*), whereas isolates from wild beet showed higher ability to cope with abiotic stresses (Zachow *et al.*, 2014). The rhizomicrobiome of wild rice was less sensitive to introduction of a fungal pathogen and an introduced pathogen had lower abundance in wild rice (Shi *et al.*, 2018). In barley, a small but significant effect of domestication was manifested mainly on the abundance of several OTUs from various taxa, rather than on single OTUs (Bulgarelli *et al.*, 2015). In contrast, domestication effects were abundant in foxtail millet (S*etaria italica*) and its wild ancestor (*S. viridis*), including effects on Betaproteobacteria and Firmicutes, which were enriched in *S. italica* (Chaluvadi & Bennetzen, 2018). In wild and domesticated *Phaseolus vulgaris*, a gradual decrease in relative abundance of members of Bacteroidetes, and an increase of members of Actinobacteria and Proteobacteria was observed from wild to modern accessions (Perez-Jaramillo *et al.*, 2017). The evolutionary history of Poaceae (maize, sorghum, wheat and teosinte) was shaping bacterial communities, and increases in phylogenetic distance were reflected in increasing differences between microbial communities (Bouffaud *et al.*, 2014).

Modern wheat cultivars are the product of at least 10,000 years of human selection. Modern bread wheat (*Triticum aestivum* L.) has an allo-hexaploid genome consisting of three subgenomes (AABBDD) resulting from hybridization events between *T. urartu* (AA genome) and a close relative to *Aegilops speltoides* (BB genome), and a later hybridization with the wild diploid *A. tauschii* (DD genome) (Pont *et al.*, 2019). *T. turgidum* ssp. *dicoccoides* and *T. turgidum* ssp. *dicoccum* are allo-tetraploid emmer wheat varieties (BBAA genomes) that are suggested as direct progenitors of modern wheat (Avni *et al.*, 2017). Domesticated emmer, *T. turgidum* ssp. *dicoccum,* was first cultivated in the fertile crescent around 10,000 BCE. Einkorn, *T. monococcum* ssp. *monococcum* and *T. monococcum* ssp. *aegilopoides* are diploid species with AA genomes, also cultivated in

the fertile crescent (Abbasov *et al.*, 2018). Spelt wheat (*T. triticum* ssp. *spelta*) represents a hexaploid (AABBDD) type of hulled wheat, characterized by adaptation to a wide range of environments (Dinu *et al.*, 2018). Several wheat traits have changed dramatically during domestication such as root architecture and exudation of primary and secondary metabolites (Beleggia *et al.*, 2016, Rascio *et al.*, 2016, Iannucci *et al.*, 2017). This may have had profound effects on root and rhizosphere microbial communities, as demonstrated in a study of plant growth-promoting bacteria associated with roots of ancient and modern wheat (Valente *et al.*, 2020). It has further been shown that modern wheat types are less dependent on arbuscular mycorrhizae than older types and that old accessions benefits more from mycorrhizal symbiosis (Kapulnik, 1991). It was suggested that this could be caused by the highly fertile conditions of soil during breeding and cultivation of modern cultivars (Hetrick *et al.*, 1993).

Understanding assembly processes of root associated microbial communities, and their diversity and ecology may enhance future breeding programs. Here, we hypothesized that the modifications of the wheat genome occurring through 10,000 years of diversification resulted in a cognate selective pressure on the root-associated microbiota. To test our hypothesis, we selected three distinct genetic groups of Triticum depicting two major events in wheat evolutionary history. The genetically oldest group that we chose was Triticum accessions of *T. monococcum* (AA genome; *Tm*) and *T. turgidum* (BBAA genome; *Tt*). To represent the wheat *T. aestivum* (genome BBAADD), we included landraces grown before the 1940s and one accession of *T. triticum* ssp. *spelta*. Four commercial cultivars currently grown in Denmark were used as representing modern wheat. We grew those accessions under field conditions in agricultural soil and compared root fungal and bacterial communities in field plots at three time points shortly after emergence of the seedlings in the autumn. To examine the importance of deterministic versus stochastic processes in the assembly of root-associated microbial communities, we employed a neutral community assembly model (Sloan *et al.*, 2006).

Methods

Soil and plant sampling

Accessions of old wheat lines were provided by NordGen, Alnarp, Sweden (https://www.nordgen.org/en/). The untreated seeds of 5 accessions of wheat ancestors (*T. turgidum* ssp. *dicoccum* (*Tt*), *T. monococcum* ssp. *monococcum* (*Tm*) and *T. monococcum* ssp. *aegilopoides* (*Tm*)), 5 accessions of landraces (*T. aestivum* ssp *aestivum*), one accession of *T. aestivum* ssp. *spelta* (*Tas*) and 4 modern wheat cultivars (*T. aestivum* ssp *aestivum*) were sown on a sandy clay loam soil in the autumn 2017 in 50 cm rows at Aarhus University, Flakkebjerg,

Denmark (55.322631°N, 11.394336°E) (Table 1). No fungicide treatments were applied. For data analysis, all accessions were categorized as modern cultivars, landraces, *Tas* or wheat ancestors, *Tt* and *Tm* (Table 1).

For each cultivar, root samples including tightly attached soil, were collected destructively in quadruplicates at roughly BBCH10 (emergence of first leaf), BBCH12 (first leaf fully unfolded) and BBCH21 (first side shoot visible) (Lancashire *et al.*, 1991) during the autumn 2017 starting from October 31 (Table S1). Each root sample was a composite of four plants, which were randomly uprooted and carefully separated from loosely attached soil.

Bulk soil samples were collected from the plough layer (0-20 cm) in between crop rows at each sampling point. In each sampling line (space between two crop rows) 10 soil sub-samples were collected, thoroughly mixed and from those, two replicate composite bulk soil samples were collected (n=10). All samples were frozen at -80^{o}C until further processing for DNA extraction.

Sample homogenization and DNA extraction

Soil and root samples were freeze-dried for 48h at -111^{o}C and 0.0026 Pa using a CoolSafe CS110-4 Pro freeze dryer (LaboGene, Denmark) and finely crushed using zirconium oxide grinding balls (10mm) and a Fast & Fluid Management shaker-SK350 (Fast & Fluid, Sassenheim Netherlands). DNA was extracted from homogenized soil and root samples using the NucleoSpin® DNA 96 Soil Kit (Macherey-Nagel GmbH & Co KG, Düren, Germany) adapted to a Biomek® FCP Laboratory Automation Workstation (Beckman Coulter™, CA, USA). Four negative controls (500µL of PCR graded water) were included per extraction plate and used for amplicon library preparation together with the samples. Concentration of extracted DNA was measured using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, MA, USA) with a Qubit dsDNA HS Assay Kit (range 0.2-100ng; Invitrogen, Life Technologies). DNA was then stored at -20^{o}C.

Amplicon library preparation and sequencing

Extracted DNA was used to produce bacterial and fungal amplicon libraries (Table S2). The fungal ITS2 region was amplified using fITS7 and ITS4 primers (Ihrmark *et al.*, 2012) and the bacterial 16S rRNA V3-V4 region was targeted using primers S-DBact-0341-b-S-17/S-D-Bact-0785-a-A-21 (Klindworth *et al.*, 2013). PCR was performed in a reaction mixture of 25 µl consisting of 1 × PCR reaction buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 1 µM of each primer, 1 U of GoTaq Flexi polymerase (Promega Corporation, Madison, USA) and 1µl of DNA template. PCR was conducted in a GeneAmp PCR System 9700 thermal cycler (Thermo Fisher Scientific, MA, USA) using 94 °C for 5 min, followed by 25 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and

a final elongation step at 72 ◦C for 10 min. For fungal amplicon library preparation, an annealing temperature of 57 °C was used as recommended (Ihrmark *et al.*, 2012). For dual indexing, primers including indexing tags were used in a PCR for 10 cycles, with the thermal cycler program as described above. In addition to dual indexing, internal barcodes of varying length were added to the forward primer for combining samples within each index combination as described earlier (Wu *et al.*, 2015, Siddique & Unterseher, 2016). After PCR, amplicon size was confirmed by visualization in a 1.5% agarose gel using SYBR staining, PCR products were pooled, precipitated and re-eluted as described earlier (Sapkota & Nicolaisen, 2018). In order to remove primers and shorter reads, the pooled DNA was separated on a 1.5 % agarose gel and amplicons of the expected size (300–450 bp) were extracted using a QIAquick Gel Extraction Kit (Qiagen, Copenhagen, Denmark). The DNA concentration of the amplicon library was evaluated using a Qubit® Flurometer (Thermo Fisher Scientific). Amplicon libraries were shipped to Eurofins MWG (Ebersberg, Germany) for sequencing on an Illumina MiSeq platform using a dual indexing strategy.

Amplicon sequencing data analysis

Quality processing and bioinformatics of the reads obtained from Illumina MiSeq were analysed as described earlier (Kudjordjie *et al.*, 2019). Briefly, paired end reads were filtered with Phred quality scores > 30 and merged with an overlapping minimum read length of 30 base pairs using VSEARCH v. 2.6 (Rognes *et al.*, 2016). Forward and reverse primers, as well as internal barcodes were trimmed and reads with less than 200 base pairs were excluded. Before clustering at 97% similarity, all reads were dereplicated and screened using VSEARCH. Taxonomy assignments for the clustered operational taxonomic units (OTUs) was done using SILVA v. 132 for bacteria and UNITE v. 8.0 for fungi in QIIME v 1.9, using assign_taxonomy.py (Caporaso *et al.*, 2010, Quast *et al.*, 2013). OTUs unassigned at kingdom level or assigned as chloroplast or mitochondrial sequences were removed from the datasets. Furthermore, samples with less than 2,000 reads were excluded from downstream analysis.

Bacterial and fungal annotation tables were analyzed in R v.3.5.2 (R Core Team, 2017), using the RStudio development environment (RStudio Team) and making use of the 'phyloseq' package (McMurdie & Holmes, 2013). The OTU tables were transformed, by either rarefaction or relative abundance, before executing diversity-based calculations. Alpha diversity was estimated using observed OTU richness and Shannon diversity measures, using the mean value from rarified OTU tables generated 100 times at a sampling depth of 3,000 reads per sample. Significant differences between diversity indices were determined using the Kruskal-Wallis rank sum test.

Bray-Curtis distance matrices were used for beta diversity analysis at OTU level using unrarefied data (all included samples with > 2,000 reads per sample) and transformed to relative abundance. Variance partitioning and significance for experimental factors were detected by PERMANOVA (R package: 'vegan'). Dissimilarity between samples (based on Bray-Curtis distances) was visualized using NMDS plots.

Community assembly model

To study the importance of selection versus neutral processes in community assembly, a modified version (Morris *et al.*, 2013) of the model described earlier (Sloan *et al.*, 2006) was applied. This neutral community assembly model (NCM) predicts the frequencies with which taxa should occur in target communities based on their abundance in the source community (also referred to as metacommunity). In short, the model predicts that abundant taxa in the source community will be observed more frequently in the target communities due to increased immigration opportunities, while rare taxa will more likely be lost in the target community due to ecological drift. The OTU table was transformed by rarefaction to 2000 reads per sample and normalization into relative abundances, before the analysis. The model fit is determined by the coupled parameter N_t*m; where N_t is the size of the community (the number of reads in the samples was considered as an estimation of the community size); m is the migration rate. The migration rate is the estimated probability that the random loss of an individual in the local community will be replaced by immigration from the source community. The fitting of the model parameters was performed in R where binomial proportion 95% confidence intervals (based on the Wilson method) around the model predictions were calculated using the 'HMisc' package (Harrell, 2016). In this study, root samples were considered as local communities, while the composition of the source community was inferred by averaging the composition of the bulk soil samples. Among the local communities, two separate groups were identified: roots communities associated with the modern opposed to old cultivars; these two analyses were performed for the fungal and bacterial communities separately. OTUs falling between the 95% confidence interval were considered to be present as a result of neutral dynamics of birth, death and immigration from the source (bulk soil); OTUs falling outside the confidence interval (and with the ratio between observed frequency and predicted frequency greater than 1.5 or lower than 0.5) were found with frequencies disproportionally higher or lower than predicted by the model based on their abundances in the soil and therefore considered to be present in the communities as a result of deterministic factors. Pearson Correlation between the frequency of the sequence variants estimated by the model and that of observed was used to determine the statistical significance of model fit. The analysis was

performed considering only OTUs shared by the bulk soil community (source) and the root community (target). In this case, all sequence variants that were detected in the root, but not in the bulk soil, and vice versa were excluded, as it is routinely done (Morris *et al.*, 2013, Kinnunen *et al.*, 2017, Vignola *et al.*, 2018). The number of OTUs shared between target and source communities and employed in the model are shown in Table 3.

Results

Amplicon sequencing quality

Sequencing of the bacterial 16S rRNA amplicon library resulted in 8,806 OTUs in 210 samples (excluding controls). Number of reads per sample ranged between 0 and ~80,000 (Figure S1). Samples with less than 2,000 reads were removed from downstream analysis (n=16). Furthermore, 504 OTUs unclassified at kingdom level and 29 sequences classified as chloroplast at family level were removed from the dataset. After clustering, the length of representative sequences of 16S V3-V4 region was 417 ± 10 bp (mean ± sd). Therefore, 11 OTUs with sequences shorter than 390 bp were eliminated from the dataset (Figure S1). Sequencing of the fungal ITS amplicon library resulted in 561 OTUs in 210 samples (excluding controls). Number of reads per samples ranged between 0 and ~150,000 (Figure S1). Samples with less than 2,000 reads were removed from the downstream analysis (n=10). Furthermore, 170 sequences not classified at kingdom level were removed from the analysis. Fourteen sequences smaller than 135 bp were removed from the dataset (Figure S1).

Rarefaction curves indicated that the number of reads sampled the variation in fungal communities whereas bacterial variation was not covered completely in our sampling strategy (Figure S2).

Community alpha diversity

As expected, bulk soil alpha diversity was always higher than the alpha diversity of the root samples (Figure 1). Alpha diversity in roots increased from BBCH10 to BBCH21. Differences in alpha diversity among genetic groups, measured as observed species richness and Shannon diversity indices, were most pronounced at BBCH 10, whereas the alpha diversity approached similar levels at BBCH21. For both bacteria and fungi, Landraces + *Tas* had the highest alpha diversity of all accessions. *Tt* generally had the lowest alpha diversity, most clearly observed in the bacterial dataset.

Microbial communities separate according to wheat evolution

Comparative analysis of modern cultivars, Landraces + *Tas*, and ancient accessions (*Tm* and *Tt*) separated the groups in NMDS plots, but differently at the three growth stages. This indicates a significant effect of wheat genotypes and BBCH on the microbial community composition (p=0.001 for each BBCH) (Figure 2, Figure S3). We observed clear separation of bulk soil microbial communities from the root communities of all wheat accessions, the latter being more dynamic over time. Although the root bacterial and fungal community composition shifted between growth stages, the separation of modern cultivars from Landraces + *Tas* and *Tt* and *Tm* was remarkable (Figure 2 and Table 2). We observed noticeable differences between the wheat genetic groups. Landraces + *Tas* clearly clustered at all growth stages while the microbial community composition of the roots of *Tt* became more similar over time and approached the other genetic groups, except the modern cultivars, indicating a stronger succession of microbial communities in the *Tt* roots than in the roots of *T. aestivum*.

Comparing the dynamics of bacterial and fungal communities, we observed a stronger selection on bacterial communities compared to fungal communities at the first growth stage (based on stronger clustering of samples). However, over time, selection in fungal communities increased. Fungal community composition in roots of ancient accessions was distinct from modern cultivars, and bacterial communities of modern cultivars were even more clearly separated from the landrace/ancient lines. Furthermore, overall fungal community composition in roots was similar to the bulk soil community composition, whereas the bacterial community composition in bulk soil clearly separated from root communities. PERMANOVA tests confirmed that both fungal and bacterial communities in soil and roots were significantly different in the total dataset ($p<0.001$), confirming our observations of the NMDS plots (Table 2). When root associated microbial data was split according to growth stage, the different wheat groups had significantly different communities ($p<0.001$; fungi at BBCH21, $p<0.01$). However, the wheat genetic groups explained a higher proportion of the variation in bacterial communities.

Taxa significantly enriched in the roots of old and modern wheat lines

For this analysis, we grouped the wheat lines into old (*Tm*, *Tt*, landraces and *Tas*) and modern lines (KWS Desanto, Substance, Sherif and Torp). We observed differences in taxa enriched in the roots of old and modern communities (Figure 3, Figure S4-S6). The most remarkable differences in bacterial enrichment could be accounted to OTUs belonging to *Acidobacteria* and *Actinobacteria* primarily in old cultivars at BBCH21, while modern cultivars enriched OTUs from *Saccharibacteria*, *Verrucomicrobia* and *Firmicutes* (Figure 3a). Furthermore, modern cultivars had higher numbers of enriched OTUs of *Bacteroidetes* at BBCH10, but this effect was absent at

BBCH21. We also observed phylum level differences of fungal taxa enriched in the roots (Figure 3b). Taxa belonging to *Ascomycota* and *Basidiomycota we*re enriched in both old and modern cultivars, but the number of enriched *Basidiomycota wa*s much higher in the old wheat lines. Although at a low number (and abundance), *Mortierellomycota, Olpidiomycota* and *Chytridiomycota* taxa *we*re solely enriched in the roots of old cultivars. At OTU level, we observed a stronger enrichment of bacterial families in the modern cultivars compared to ancient lines (Figure S5a). Interestingly, similar patterns were not observed for the fungal communities (Figure S5b). Modern wheat cultivars strongly enriched for OTUs belonging to *Rhizobiaceae*, *Flavobacteriaceae* and *Pedobacter*. The most significantly enriched family in the communities of the old lines was *Sphingomonadaceae* (Figure S6a). *Microdochium*, *Neoascochyta*, *Sporobolomyces* and *Fusarium* were enriched in modern cultivars whereas basidiomyceteous yeasts such as *Filobasidium, Holtermaniella* and *Psathyrella*, and the ascomycete *Aureobasidium* were enriched in the old lines (Figure S6b). Surprisingly, we observed that genera within *Basidiomycota* were more frequently enriched (and with higher significance) in old lines, while *Ascomycota* dominated in the modern cultivars.

What drives the community assembly in the roots of wheat?

Bacterial communities from both modern and old wheat accessions showed very strong correlation with the neutral model (NCM) (Sloan *et al.*, 2006) when the bulk soil community was considered as the source (Figure 4). The model explained 75% and 78% (Pearson correlation) of the observed variation in the frequencies of OTUs present in the local bacterial communities associated with modern and old lines, respectively (Table 3). Fungal communities also correlated strongly with the model: a Pearson correlation value of 0.86 was observed for the modern community and 0.87 for the ancient community (Table 3). The immigration rates for the bacterial and fungal communities were estimated from the model (Ntm value used to fit the model with the observations divided by 2000 reads): 7.8% and 7.6% of the deaths happening in bacterial communities from modern and old accessions were replaced by OTUs from the source. Lower values were observed for the fungal communities: 3.0% and 4.8% for the modern and old community, respectively. Among the old cultivars, we distinguished two further subsets: *T. aestivum* landraces including *Tas*, and *T. monococcum* and *T. turgidum* lines. The migration rates for these two subsets of bacterial communities were 9.1% and 6.1%, respectively. Results suggest that among old accessions, *Tm* and *Tt* lines created a more isolated environment where fewer deaths happening within the local community were replaced by immigrants from the source community, compared to the environment created by the *T.aestivum* landraces.

Discussion

The goal of this study was to identify potential evolutionary 'post-domestication breeding-effects' in wheat by comparing root microbial communities in three distinct genetic groups of Triticum depicting major events in the evolutionary history of wheat: modern cultivars, landraces and ancestors of wheat. Breeding for higher yields in high-input agriculture may inadvertently have led to depletion of beneficial microbial taxa associated with plant roots. For example, it was found that ancient wheat varieties were more capable of interacting with beneficial plant growth-promoting rhizobacteria (Valente *et al.*, 2020). Generally, the magnitude of genotype effects on microbial communities is significant but small (Peiffer *et al.*, 2013, Sapkota *et al.*, 2015, Wagner *et al.*, 2016). While a number of studies have investigated genotype effects in modern crop plants, fewer studies have specifically investigated long-term evolutionary effects by comparing modern crops and ancestors of those crops (Zachow *et al.*, 2014, Bulgarelli *et al.*, 2015, Leff *et al.*, 2017, Chaluvadi & Bennetzen, 2018, Brisson *et al.*, 2019). In the present study, we demonstrated effects of long-term breeding processes on root-associated microbial diversity, and we demonstrated that bacterial and fungal communities differed in a transect of evolution between the different groups of wheat: modern cultivars, landraces (including *T. triticum* ssp. *spelta*) and wheat ancestors (*T. turgidum* ssp. *dicoccum*, *T. monococcum* ssp. *monococcum* and *T. monococcum* ssp. *aegilopoides*). We were able to identify enriched bacterial and fungal taxa in old and modern wheat accessions, and we demonstrated that modern cultivars followed a neutral community assembly model to a higher degree than old lines, suggesting that the old lines posed a stronger selection on microbial community assembly.

Bacterial and fungal alpha diversity in bulk soil was higher than in root-associated communities, as has also been observed in numerous other studies, e.g. (Berendsen *et al.*, 2012). Both bacterial and fungal alpha diversity in root samples were generally lowest at BBCH10 and then dramatically increased and approached similar diversity levels at BBCH21. Similarly, rapid colonization of rice roots was demonstrated even after a few days following transplantation of seedlings to soil, and succession of communities that stabilized after 2 weeks of plant growth (Edwards *et al.*, 2015). Overall, our results show that there is a rapid initial microbial colonization phase from bulk soil to the root, and we speculate that this period is of high importance in shaping community structures in plant roots, also at the later stages of growth.

Bacterial colonization of roots of modern cultivars occurred faster than in wheat ancestors (*Tm* and *Tt*), indicating that wheat ancestors subject stronger selection on bacteria at the initial growth stages. On the other hand, the lowest diversity of fungal communities was observed in modern

cultivars at BBCH10. We speculate that one reason for this difference between bacterial and fungal communities could be that focus in plant breeding has been on fungal pathogens and that the plant immune system has been tuned to prevent fungal invasion. Thus, modern cultivars may be more selective towards fungi from the earliest stages of root development, inhibiting rapid stochastic colonization. The highest diversities of both bacteria and fungi were observed in the wheat landraces, as was also observed in other studies (Germida, 2001, Szoboszlay *et al.*, 2015). Landraces have been adjusted to agricultural settings during selection, whereas wheat ancestors *Tm* and *Tt* have been harvested from native soil and are thus not adjusted to the microbial communities found in modern agricultural soil. At the same time, landraces were bred in low-input soil in opposite to modern cultivars that have been bred in high-input soil, promoting lower dependency on microbial interactions in the root.

Both fungal and bacterial communities differentiated according to bulk soil and wheat type (modern, landrace + *Tas* or ancient (*Tm* and *Tt*)) in NMDS plots. Bulk soil communities separated from root communities over time, with the most remarkable separation observed at BBCH21. We observed a stronger selection of bacterial and fungal communities at the later growth stages based on the more distinct clustering of samples at BBCH21. Landraces and ancient lines of wheat clustered more distinctly at later stages, which could indicate a stronger selection during the colonization of ancient lines, as was also observed in a recent study (Hassani *et al.*, 2020). This study suggested stochastic processes are of higher importance in modern wheat lines compared to ancient lines and that domestication could have entailed less selective constraints on plant traits that contribute to microbial assembly. Bacterial communities clustered more distinctively at BBCH10 and BBCH12 compared to fungal communities suggesting a stronger selection of bacteria at the initial stages of colonization. Fungal and bacterial communities in the ancient wheat accessions clearly separated from modern cultivars already at BBCH10, showing that effects of breeding are manifested already at the initial growth stages of the wheat seedlings. Also of notice, both bacterial and fungal communities in modern cultivars separated from the communities in ancient and landrace accessions.

Several bacterial and fungal taxa were enriched in old or modern accessions. We noted that more members of *Acidobacteria* and *Actinobacteria* were enriched in the old accessions, while more OTUs from *Candidatus Saccharibacteria*, *Verrucomicrobia* and *Firmicutes* were enriched in the modern cultivars. Interestingly, most of these OTUs were predominantly enriched in BBCH21. *Acidobacteria, Verrucobacteria* and *Actinobacteria* are typically found in high abundance in the soil and rhizosphere, and plant roots selectively enrich for certain groups of *Acidobacteria* and *Verrucobacteria* (Nunes da Rocha *et al.*, 2013)*. Actinobacteria* and *Firmicutes* are associated with

soil suppressiveness (Mendes *et al.*, 2011) and may thus be recruited as antagonists against soil-borne pathogens. At a lower taxonomic level, *Rhizobiaceae, Flavobacteriaceae* and *Pedobacter* were all enriched in modern cultivars. Interestingly, these taxa have all been suggested to have plant beneficial properties and to be enriched in the roots or rhizosphere of cereals (Yin *et al.*, 2013, Poole *et al.*, 2018, Carrion *et al.*, 2019). Further corroborating this, a comparative study of microbiomes of cultivated sugar beet and its wild ancestor *Beta vulgaris* ssp. *maritima* found that a higher proportion of isolates that were active against phytopathogens in domesticated sugar beet (Zachow *et al.*, 2014). Likewise, higher amounts of *Flavobacteriaceae* in the roots and rhizosphere of a modern barley cultivar compared to the ancestor *Hordeum vulgare* ssp. *spontaneum* were found (Bulgarelli *et al.*, 2015). We observed enrichment of several known fungal wheat pathogens such as *Fusarium*, *Neoascochyta* and *Microdochium* in the modern cultivars. *Fusarium* was also enriched in roots of modern cultivars of sunflower compared to non-domesticated sunflower (Leff *et al.*, 2017). Whether enrichment of certain putative fungal pathogens in modern cultivars represents a general trend is unknown, but it is tempting to speculate that domesticated wheat may have lost the ability to prevent certain soil-borne pathogens, as those pathogens are not important in modern agricultural systems including efficient crop rotations.

Both bacterial and fungal communities of the roots of wheat fitted the neutral community assembly model very well suggesting that stochastic factors of birth, death and immigration from the bulk soil are largely driving the composition of both bacterial and fungal communities associated with wheat roots. The migration rates for landraces + *Tas* were higher compared to the ancient lines *Tm* and *Tt* (9.1% vs. 6.1%). This suggests that *Tm* and *Tt* lines created a more isolated environment from the soil compared to the environment created by landraces. Rossmann *et al.* (2020) observed stronger interactions in landraces with their soil microbiomes compared to modern wheat. Hassani *et al.* (2020) found a higher stochasticity in the assembly of microbial communities in modern wheat compared to the two ancestors *T. boeoticum* and *T. urartu*. With a lower immigration rate, we also expected lower alpha diversity, and indeed, we observed that *Tm* and *Tt* had the lowest alpha diversity. The high diversity of the soil was lost in the local root communities and the more the local communities were isolated (low immigration rates) the less diversity we observed (Rosindell *et al.*, 2011).

The results of our study suggest that changes have occurred during the process of breeding in the ability of wheat to assemble fungal and bacterial root communities. Whether these changes are a result of the selection for certain wheat traits under high nutrient availability remains an open question. It also remains to be elucidated whether such selection has led to changes in the ability

of wheat to cope with environmental stresses, to take up nutrients from the soil more efficiently and to avoid pathogens in the root more effectively. However, indications of less dependency in modern wheat on microbial interactions have emerged. Similarly, wheat dependence of mycorrhizal interactions was higher in landraces compared to ancestors and modern lines, and responsiveness to mycorrhizal colonization was lower in modern cultivars (Schmidt *et al.*, 2016). In conclusion, we have demonstrated remarkable effects of human-induced evolution in wheat. We have shown that microbial diversity in wheat roots grown in agricultural soil is highest in landraces bred in low-input agricultural systems. We also demonstrated that the first period after emergence of seedlings is crucial for microbial colonization of roots. Several taxa were enriched exclusively in modern or old wheat lines. Communities in both modern and old wheat lines followed the predictions of the neutral community assembly model, but ancient accessions (*Tm* and *Tt*) created more isolated environments.

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Availability of data and materials

All amplicon sequencing files from this study were deposited in the NCBI sequence read archive under the accession number PRJNA611715.

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590 **Figures and tables**

591 **Table 1.** Triticum accessions used in this study.

592 **Table 2.** Permutation analysis of variance (PERMANOVA) of root-associated bacterial and fungal
593 communities in the different wheat genotypes (modern cultivars, Landraces + *Tas* and ancient
594 lines). Adonis tests were based on Bray-Curtis distance matrices using 1,000 permutations. After
595 the initial analysis of the total dataset, bulk soil samples were removed from the dataset, and the
596 remaining data was split into growth stage.

597 **Table 3**. Number of OTUs present in the target communities (roots), in the source community
598 (bulk soil) and the OTUs shared between the communities and retained for the NCM analyses.
599 Pearson correlations and migration rates are reported.

Figure 1. Upper panels: diversity indices for bacterial communities rarefied to 3,000 reads (observed richness (first 3 panels) and Shannon Diversity Index (last 3 panels)) calculated at OTU level. Lower panels: diversity indices for fungal communities rarefied to 3,000 reads (observed richness (first 3 panels) and Shannon Diversity Index (last 3 panels). BBCH10 (emergence of first leaf), BBCH12 (first leaf fully unfolded) and BBCH21 (first side shoot visible) (Lancashire et al., 1991).

Figure 2. Nonmetric multidimensional scaling (NMDS) ordination of bacterial (A) and (B) fungal community composition in bulk soil and roots of modern, landrace + Tas, and Tm and Tt wheat accession at each sampled growth stage based on Bray-Curtis distances. BBCH10 (emergence of first leaf), BBCH12 (first leaf fully unfolded) and BBCH21 (first side shoot visible) (Lancashire et al., 1991).

Figure 3. OTUs belonging to bacterial (A) and fungal (B) phyla that are differentially enriched in the roots of old cultivars (log2Fold change <0) or modern cultivars (log2Fold change >0). "Old accessions" depicted in this figure groups the wheat ancestors *Tm* and *Tt*, landraces and *Tas*. "Modern cultivars" groups the commercial cultivars KWS Desanto, Substance, Sheriff and Torp. BBCH10 (emergence of first leaf), BBCH12 (first leaf fully unfolded) and BBCH21 (first side shoot visible) (Lancashire et al., 1991).

Figure 4. Fit of the predicted neutral model (NCM) to observed bacterial (top panels) and fungal community (bottom panels) assembly at OTU level. The predicted occurrence frequencies for OTUs shared between bulk soil and roots in modern (A, C) and old (B, D) wheat accessions and the predicted occurrence frequencies. For the comparison, KWS Desanto, Substance, Sheriff and Torp are classified as "modern" and the rest as "old" cultivars (Table 1). OTUs that occur significantly more frequently than predicted by the model are shown in green, while those that occur less frequently than predicted are shown in red. Dashed lines represent 95% confidence intervals around the model prediction (black line), m = immigration rate, ρ=Pearson correlation.

Supplementary figures and tables

Table S1. Sampling times. Sampling was at BBCH 10, 12 and 21. Note that accessions Metalla, TRI 1774 and Substance were sampled on later time points due to later development. BBCH10 (emergence of first leaf), BBCH12 (first leaf fully unfolded) and BBCH21 (first side shoot visible) (Lancashire et al., 1991).

Table S2. Sampling and sequencing strategy. Number of bacterial and fungal reads are shown for each sample.

Figure S1. Overview of the bacterial 16S amplicon (top panels) and fungal ITS amplicon (bottom panels) sequencing quality: (A) number of reads per sample and (B) the length of unique amplicon sequences. Red lines represent cut-off values for removing low quality samples and sequences.

Figure S2. Rarefaction curves for (A) bacterial 16S rRNA amplicon library and (B) fungal ITS amplicon library.

Figure S3. Nonmetric multidimensional scaling (NMDS) ordination of bacterial and fungal community composition in bulk soil and roots of the different accessions at each growth stage based on Bray-Curtis distances. BBCH10 (emergence of first leaf), BBCH12 (first leaf fully unfolded) and BBCH21 (first side shoot visible) (Lancashire et al., 1991).

Figure S4. Comparison of the frequency of observation of unique genera of bacteria (A) and fungi (B) in the roots of modern and old ('monococcum' = *Tm*, 'turgidium' = *Tt* and 'aestivum' = landraces) wheat varieties.

Figure S5. Fungal genera significantly enriched in the roots of old accessions (log2 fold change <0) when compared to the modern cultivars (log2 fold change >0). "Old accessions" depicted in this figure groups the wheat ancestors *Tm* and *Tt*, landraces and *Tas*. "Modern cultivars" groups the commercial cultivars KWS Desanto, Substance, Sheriff and Torp. BBCH10 (emergence of first leaf), BBCH12 (first leaf fully unfolded) and BBCH21 (first side shoot visible) (Lancashire et al., 1991).

Figure S6. Comparison of unique bacterial (A) and fungal (B) OTUs enriched differentially in ancient and modern cultivars. "Ancient accessions" depicted in this figure groups the wheat ancestors *Tm* and *Tt*, the chosen landraces and *Tas*. "Modern cultivars" groups the commercial cultivars KWS Desanto, Substance, Sheriff and Torp.

Table 1. Triticum accessions used in this study.

Name	**Accession**	**Genome**	**Category**	**Scientific name**	**Remarks**
Metalla	NGB22783	AA	*T. monococcum* (*Tm*)	*T. monococcum* ssp *monococcum*	Cultivated Einkorn
Einkorn	NGB22756				
TRI 1774	NGB10931			*T. monococcum* ssp *aegilopoides*	Wild Einkorn
KVL 2379	NGB4802				

Emmer	NGB8190	BBAA	*T. turgidum* (*Tt*)	*T. turgidum* ssp *dicoccon*	Emmer
Hallets	NGB8967	BBAADD	Landrace	*T. aestivum* ssp *aestivum*	Known in UK before 1900
ALS	NGB4770				Known in Denmark in the 1940s
Kolbe	NGB7032				Known before 1900.
Lys Østpreussisk Hvede	NGB8979				Known before 1900
Square Head II	NGB5147				Known from the 1870s
Oberkulmer (selection)	NGB22516		Spelt (*Tas*)	*T. aestivum* ssp. *spelta*	Spelt
KWS Desanto	N/A		Modern cultivars	*T. aestivum* ssp *aestivum*	Susceptible to *Blumeria graminis* and *Zymoseptoria tritici*
Substance	N/A				Resistant to *Blumeria graminis* and *Zymoseptoria tritici*, susceptible to *Puccinia striiformis*
Sheriff	N/A				Resistant to *Blumeria graminis*, *Zymoseptoria tritici* and *Puccinia striiformis*
Torp	N/A				Susceptible to *Blumeria graminis* and *Zymoseptoria tritici*

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Table 2. Permutation analysis of variance (PERMANOVA) of root-associated bacterial and fungal communities in the different wheat genotypes (modern cultivars, Landraces + *Tas* and ancient lines). Adonis tests were based on Bray-Curtis distance matrices using 1000 permutations. After the initial analysis of the total dataset, bulk soil samples were removed from the dataset, and the remaining data was split into growth stage.

Dataset	**Factor**	**Df**	**Bacteria (R^2)**	**Fungi (R^2)**
Total	Bulk soil vs root	1	0.13***	0.06***
Roots	Triticum genetic group	3	0.11***	0.08***
	Growth stage (BBCH)	2	0.06***	0.05***
	Triticum genetic group * BBCH	6	0.12***	0.05***
Roots BBCH10	Triticum genetic group	3	0.23***	0.13***
Roots BBCH12	Triticum genetic group	3	0.17***	0.17***
Roots BBCH21	Triticum genetic group	3	0.34***	0.11**

668 **Table 3**. Number of OTUs present in the target communities (roots), in the source community
669 (bulk soil) and the OTUs shared between the communities and retained for the NCM analyses.
670 Pearson correlations and migration rates are reported.
671

	Bacteria		Fungi	
	Modern	Ancient	Modern	Ancient
Target OTUs	4254	5846	318	365
Source OTUs	4539	4539	311	311
Shared OTUs	3146	3883	285	305
Pearson correlation	0.75	0.78	0.86	0.87
Migration rate (%)	7.8%	7.6%	3.0%	4.8%

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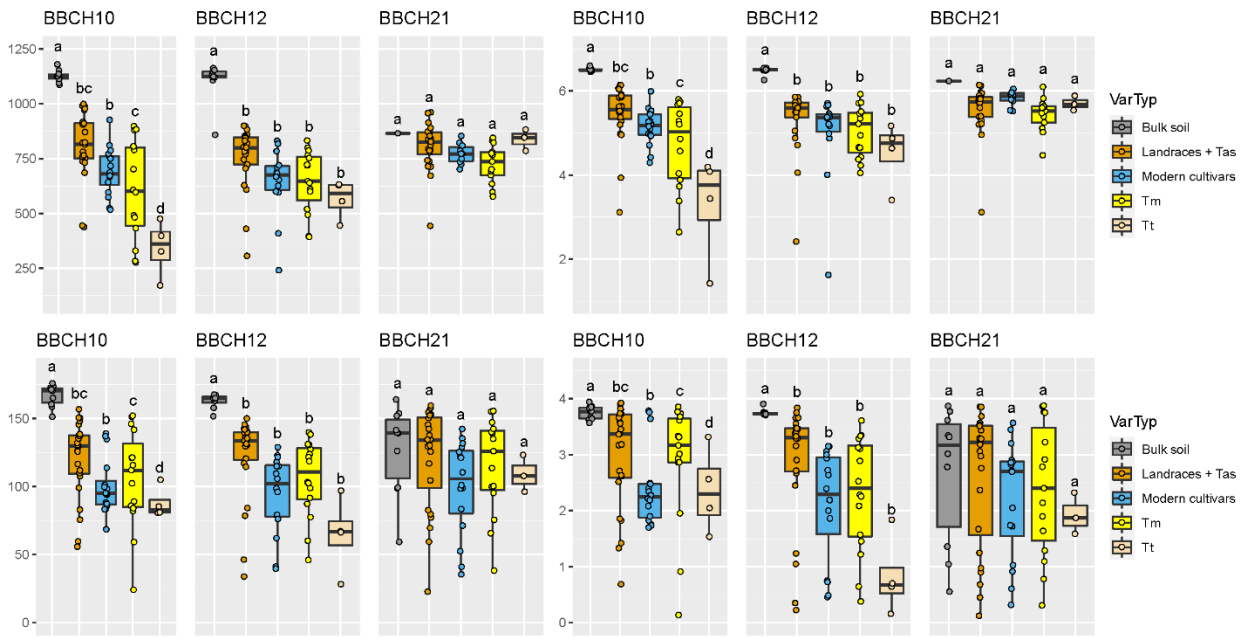

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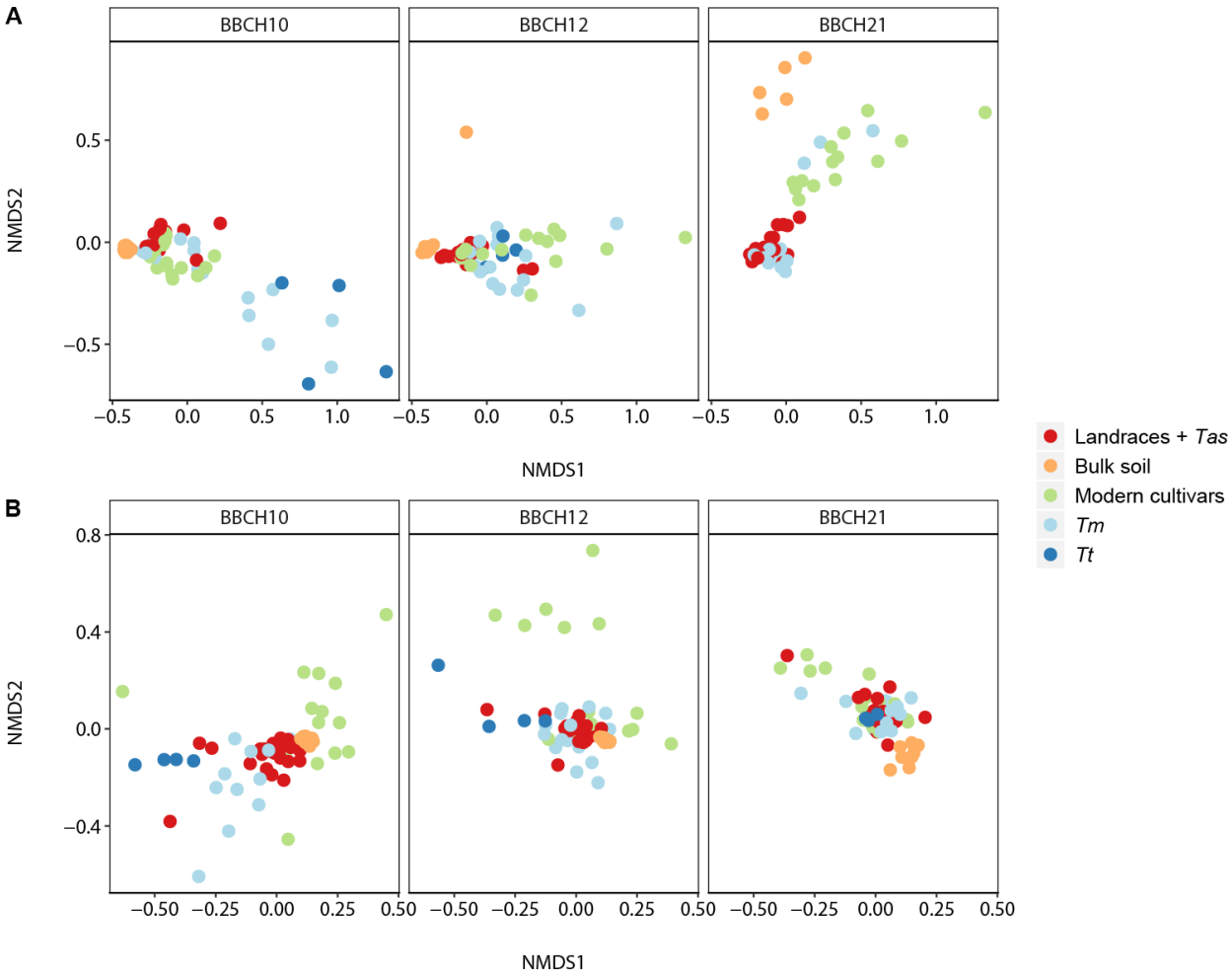

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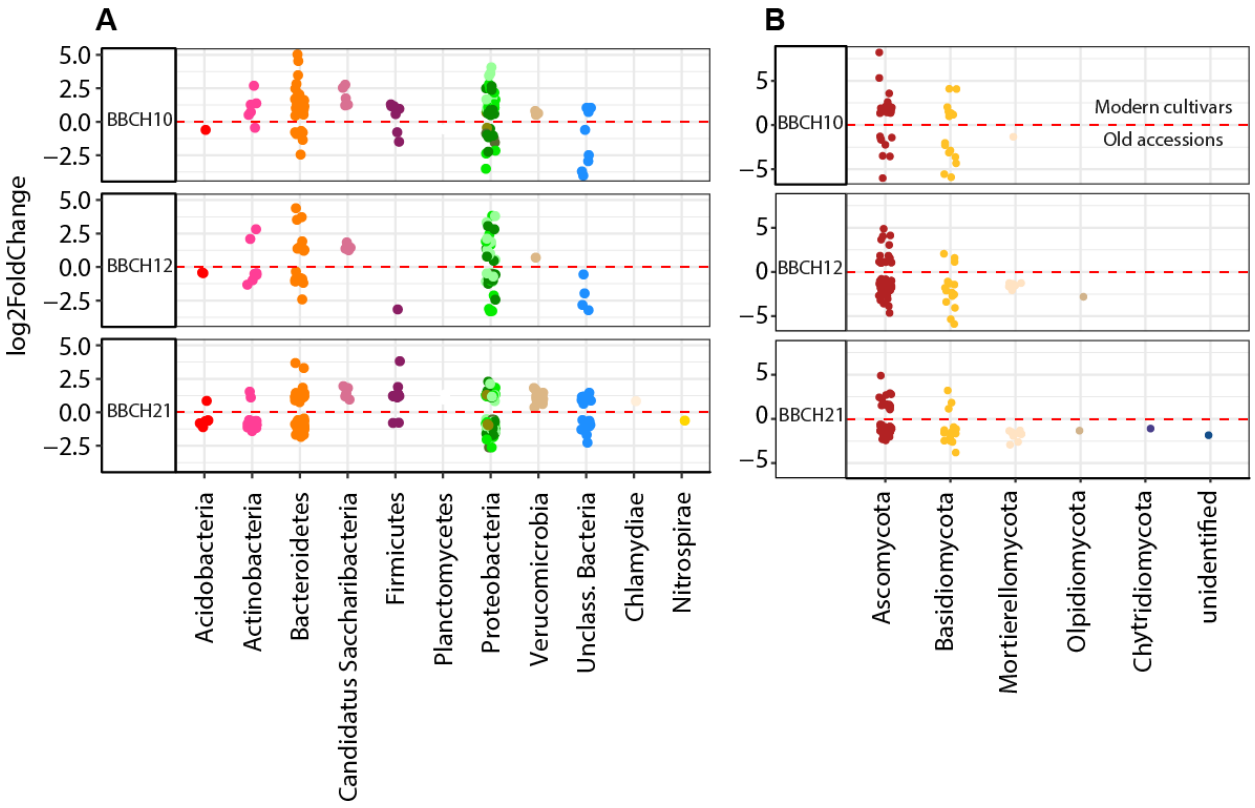

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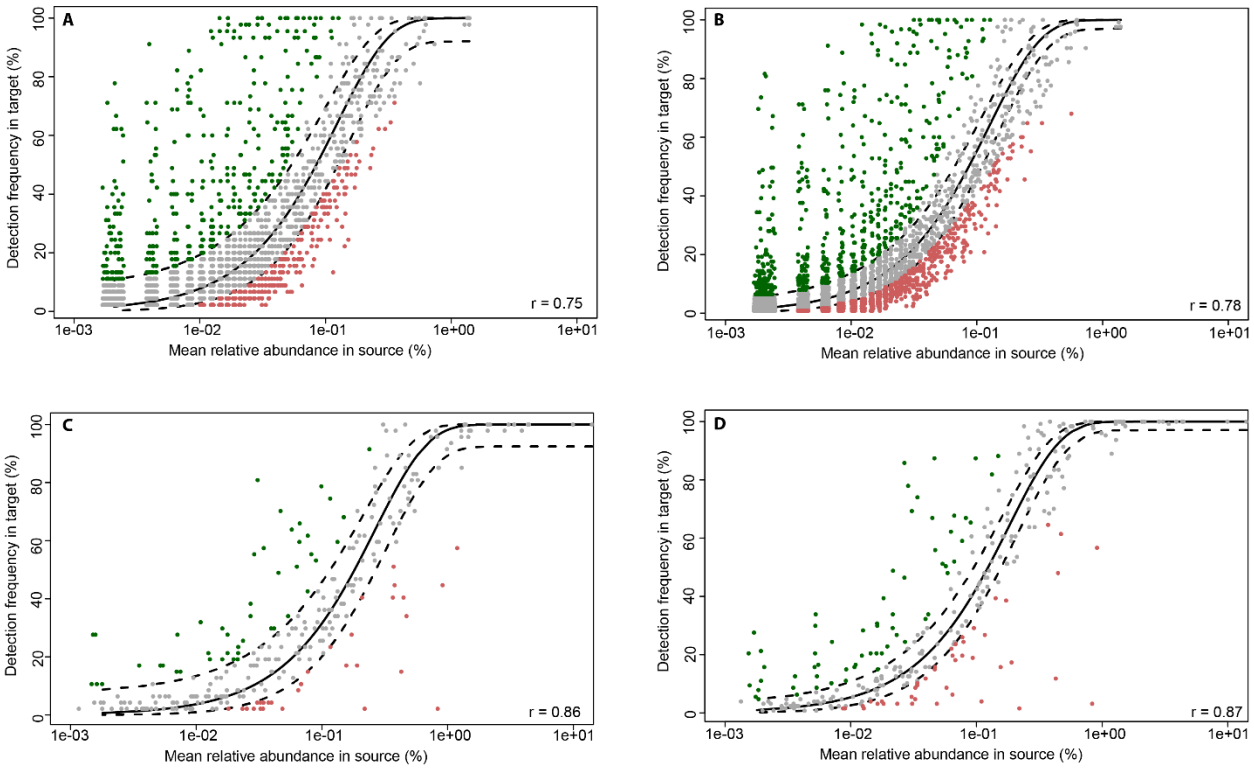

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Supplemental tables

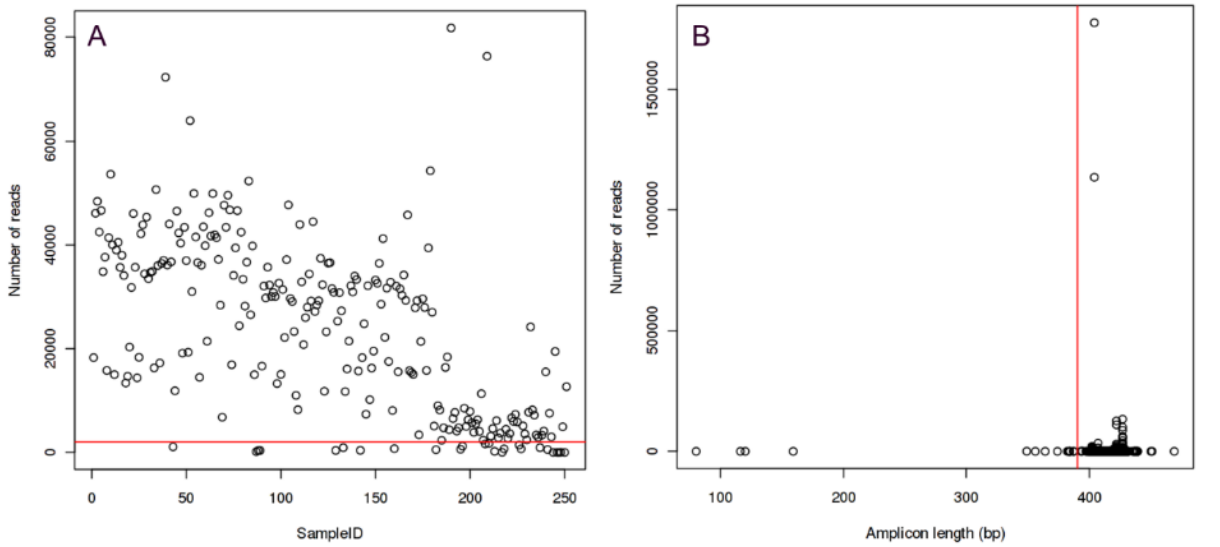
Table S1. Sampling times. Sampling was at BBCH 10, 12 and 21. Note that accessions Metalla, TRI 1774 and Substance were sampled on later time points due to later development.

Accession	Name	BBCH 10	BBCH 12	BBCH 21
Bulk soil	Bulk soil	Oct 31	Nov 9	Nov 29
Nordgen NGB22783	Metalla (selection)	Nov 8	Nov 16	Dec 6
Nordgen NGB22756	Einkorn wheat, Søtofte	Oct 31	Nov 10	Nov 29
Nordgen NGB10931	TRI 1774	Nov 6	Nov 16	Dec 6
Nordgen NGB4802	KVL 2379	Oct 31	Nov 10	Nov 29
Nordgen NGB8190	Emmer	Oct 31	Nov 10	Nov 29
Nordgen NGB8967	Hallets	Oct 31	Nov 10	Nov 29
Nordgen NGB4770	ALS	Oct 31	Nov 10	Nov 29
Nordgen NGB7032	Kolbe	Oct 31	Nov 10	Nov 29
Nordgen NGB8979	Lys Østpreussisk Hvede	Oct 31	Nov 10	Nov 29
Nordgen NGB5147	SquareHead II	Oct 31	Nov 10	Nov 29
Nordgen NGB22516	Oberkulmer (selection)	Oct 31	Nov 10	Nov 29
N/A Modern cultivar	KWS Desanto	Oct 31	Nov 10	Nov 29
N/A Modern cultivar	Substance	Nov 8	Nov 16	Dec 6
N/A Modern cultivar	Sheriff	Oct 31	Nov 10	Nov 29
N/A Modern cultivar	Torp	Oct 31	Nov 10	Nov 29

Table S2. Sampling and sequencing strategy. Number of bacterial and fungal reads are shown for each sample.

Excel sheet

Supplementary figures



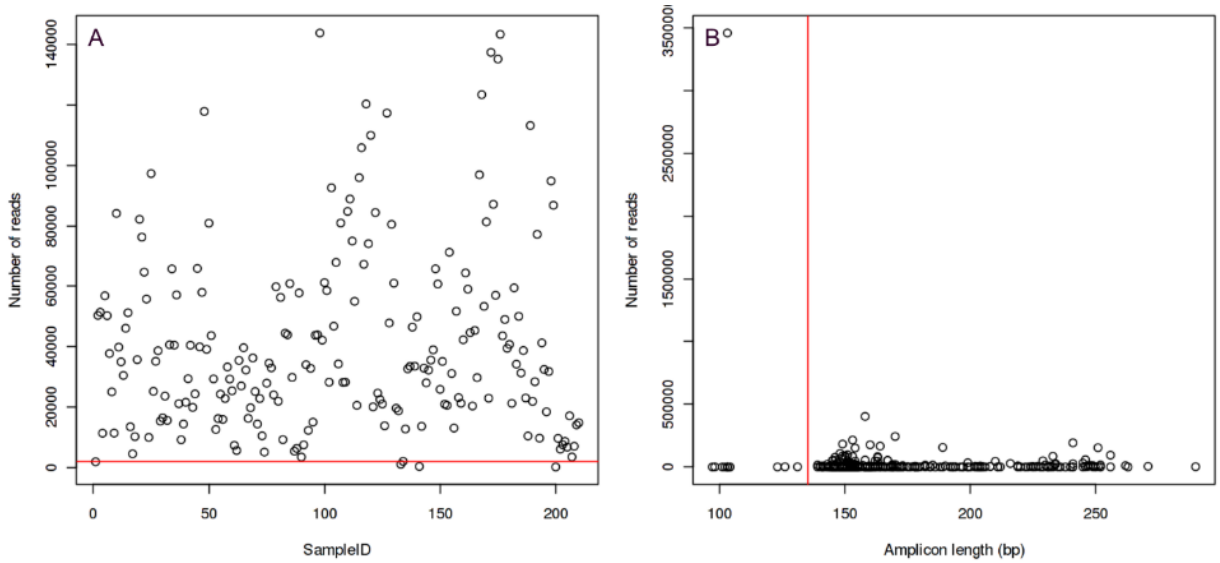
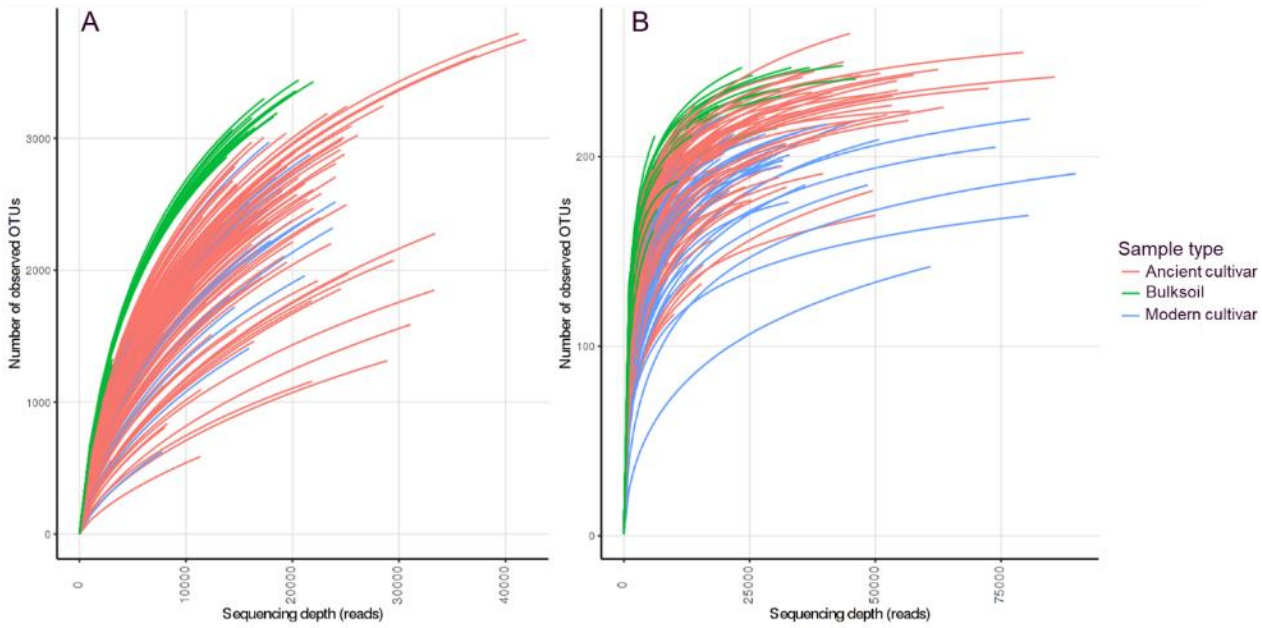


Figure S1. Overview of the bacterial 16S amplicon (top panels) and fungal ITS amplicon (bottom panels) sequencing quality: (A) number of reads per sample and (B) the length of unique amplicon sequences. Red lines represent cut-off values for removing low quality samples and sequences.



732

733 **Figure S2.** Rarefaction curves for (A) bacterial 16S rRNA amplicon library and (B) fungal ITS
734 amplicon library.

735

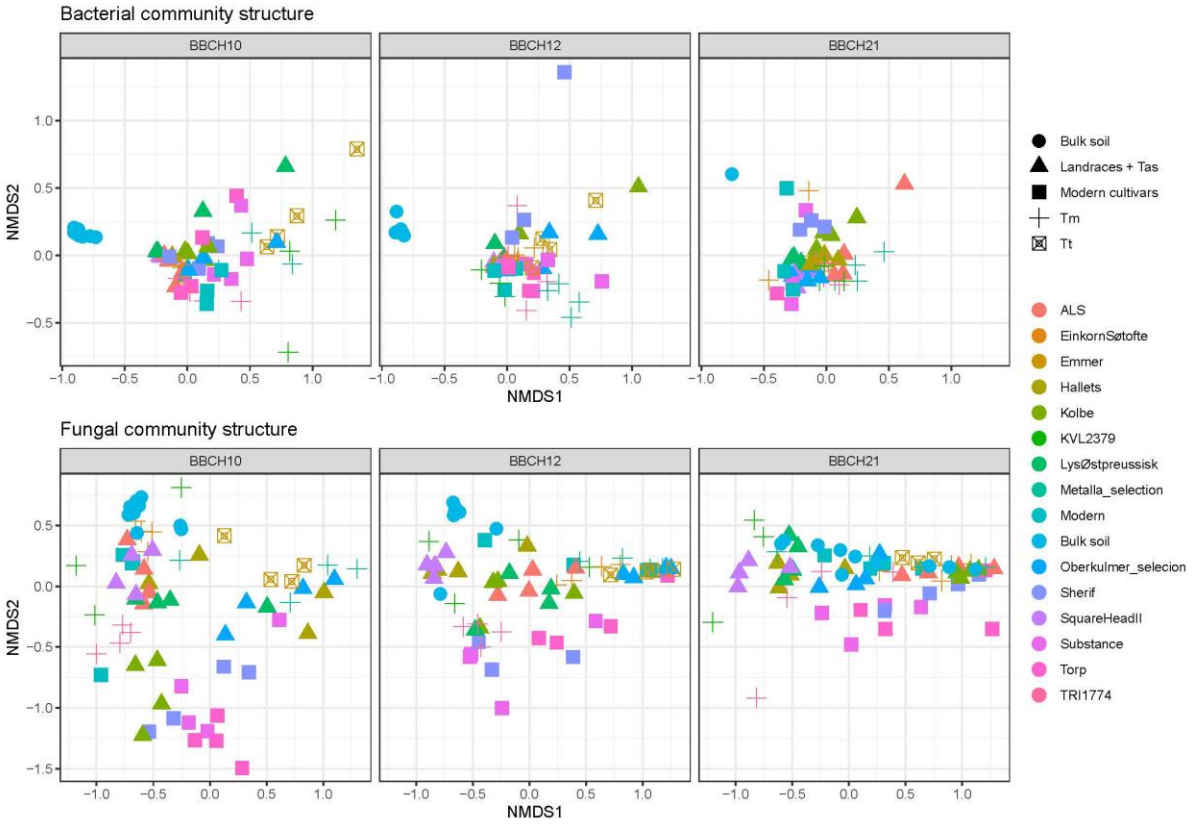


Figure S3. Nonmetric multidimensional scaling (NMDS) ordination of bacterial and fungal community composition in bulk soil and roots of the different accessions at each growth stage based on Bray-Curtis distances. BBCH10 (emergence of first leaf), BBCH12 (first leaf fully unfolded) and BBCH21 (first side shoot visible) (Lancashire et al., 1991).

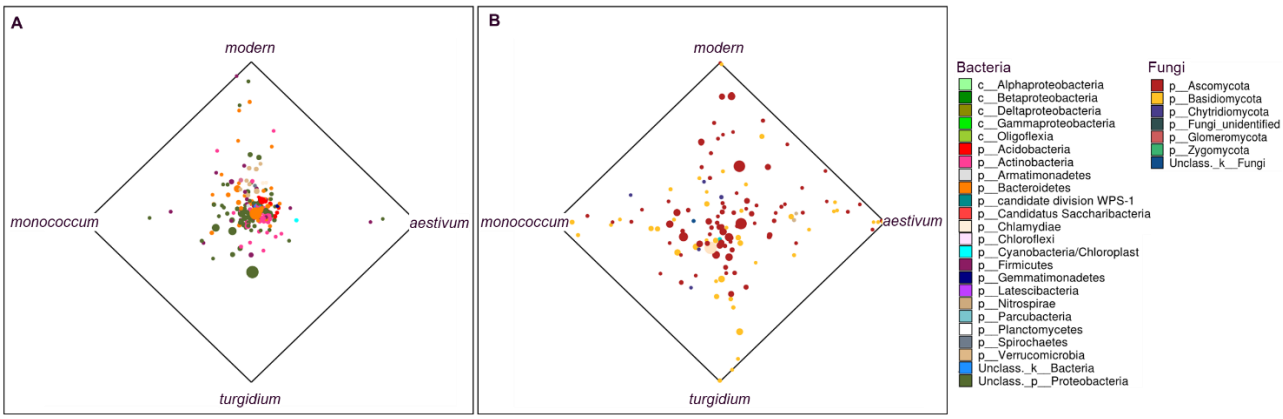


Figure S4. Comparison of the frequency of observation of unique genera of bacteria (A) and fungi (B) in the roots of modern and old ('monococcum' = *Tm*, 'turgidium' = *Tt* and 'aestivum' = landraces) wheat varieties.

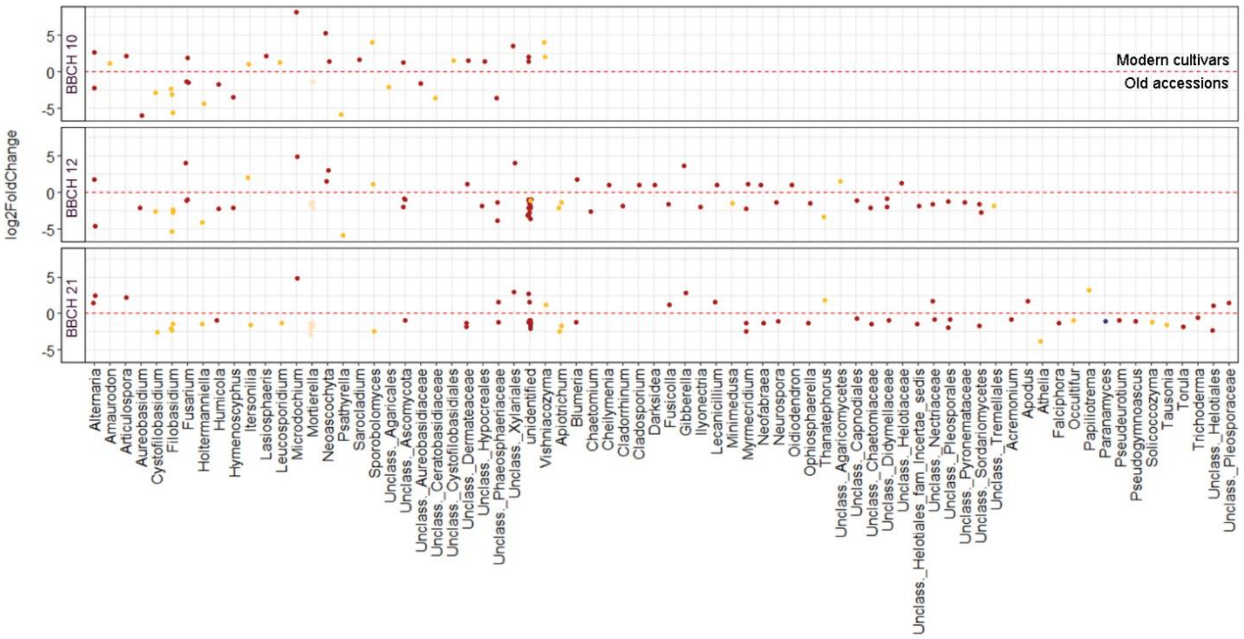


Figure S5. Fungal genera significantly enriched in the roots of old accessions (log2Fold change <0) when compared to the modern cultivars (log2Fold change >0). “Old accessions” depicted in this figure groups the wheat ancestors *Tm* and *Tt*, landraces and *Tas*. “Modern cultivars” groups the commercial cultivars KWS Desanto, Substance, Sheriff and Torp.

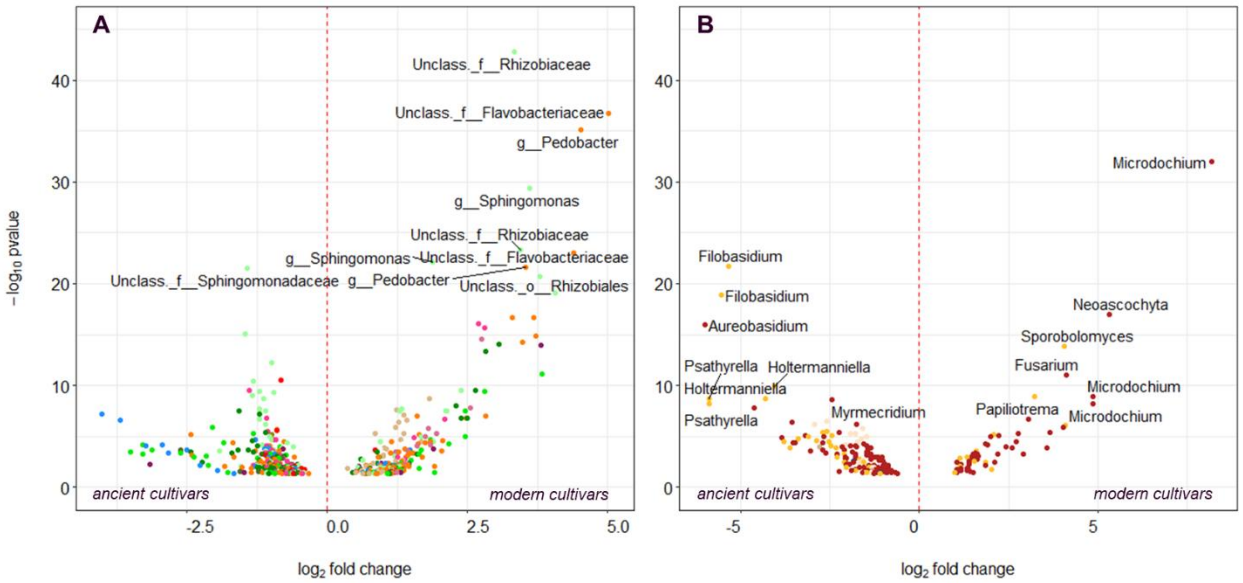


Figure S6. Comparison of unique bacterial (A) and fungal (B) OTUs enriched differentially in ancient and modern cultivars. “Ancient accessions” depicted in this figure groups the wheat ancestors *Tm* and *Tt*, the chosen landraces and *Tas*. “Modern cultivars” groups the commercial cultivars KWS Desanto, Substance, Sheriff and Torp.