Nitrogen Availability Modulates the Host Control of the Barley Rhizosphere Microbiota.

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Abstract

Background:

Since the dawn of agriculture, human selection on plants has progressively differentiated input-demanding productive crops from their wild progenitors thriving in marginal areas. Barley (*Hordeum vulgare*), the fourth most cultivated cereal globally, is a prime example of this process. We previously demonstrated that wild and domesticated barley genotypes host distinct microbial communities in their rhizosphere. Here we tested the hypothesis that microbiota diversification is modulated by, and in response to, nitrogen (N) application in soil and we assessed the impact of microbiota composition on plant growth.

Methods:

We grew two wild (*H. vulgare* ssp. *spontaneum*) and a modern domesticated (*H. vulgare* ssp. *vulgare*) barley genotypes in an agricultural soil amended with and without nitrogen (N) inputs. By using a two-pronged 16S rRNA gene survey and a shotgun metagenomics approach, we determined the impact of N application on the taxonomic composition of the barley microbiota as well as the functional diversification of microbial communities exposed to limiting nitrogen supplies. In parallel, we used metagenomics reads to reconstruct genomes of individual bacterial members of the microbiota. Finally, we implemented a plant-soil feedback experiment to assess the microbiota's contribution to plant growth.

Results:

Rhizosphere profiles were distinct from unplanted soil controls and displayed a significant, plant-mediated, N application-dependent taxonomic diversification which is maximised under N-limiting conditions. Strikingly, this diversification mirrors a metabolic specialisation of the barley microbiota, with functions implicated in nitrogen and sulphur metabolism enriched in a wild genotype as opposed to the RNA and cell capsule metabolisms enriched in a modern genotype. We reconstruct 28 high-quality individual bacterial genomes with a bias for Bacteroidetes and Proteobacteria, which are among the taxa differentially recruited between wild and modern genotypes. A plant-soil feedback experiment revealed that modern plants exposed to heat-sterilised soils grew less compared to plants maintained in untreated soils, although this difference was significant only for plants exposed to the wild barley microbiota.

Conclusions:

Our results point at nitrogen availability as a modulator of the structural and functional configuration of the rhizosphere bacterial communities and suggest a limited, but

significant, contribution of the wild barley microbiota to plant growth. This knowledge will contribute to devise strategies to enhance sustainable crop production.

Keywords:

Barley, Rhizosphere, Microbiota, Nitrogen, Domestication, 16S rRNA gene, shot-gun metagenomics, metagenome-assembled genomes, plant-soil feedback.

Background

In the past 50 years global agricultural production has increased tremendously, mainly driven by the introduction of novel crop varieties and anthropic inputs, such as the use of chemical fertiliser and pesticides. These innovations, often referred to as "The Green Revolution", undisputedly reduced hunger and improved nutrition worldwide [1]. However, it is equally undisputed that these innovations came with environmental costs, represented by the detrimental effects of agricultural practices on the environment (e.g., pollution of natural resources) and an economic burden for farmers globally, in particular in underdeveloped and developing areas [1].

This is particularly true for Nitrogen (N) applications in staple crop like cereals, which do not engage in symbiotic relationships with nitrogen-fixing microbes: it has been estimated that the global application of mineral nitrogen fertilisers is approximately 67 Tg per year, representing almost half of the total N applications to cropland [2]. By 2050 crop demand is expected to surge to 100-110% of present days figures, and this will exert further pressure on nitrogen fertilisation [3]. However, nitrogen fertilisation is a notoriously inefficient process: approximately 50% of the N fertiliser applied to crops is not absorbed by plants but instead is lost either in the atmosphere or in groundwater [4]. These losses are dependent on soil characteristics and driven by soil microbes, predominantly bacteria and archaea, capable of converting N forms present in organic and inorganic fertilisers into more soluble (e.g., nitrate) or volatile (e.g., nitrous oxide) forms [5]. These processes invariably increase costs of production and the pollution of water bodies and the atmosphere. At the same time, free-living microbes capable of mineralising nitrogen from soil organic matter and/or converting atmospheric di-nitrogen into ammonia (i.e., diazotrophs) can offset, at least in part, these losses by contributing with a net input of 'phytoavailable N' in soil [6]. Therefore, a better understanding of the functions provided by microbes thriving at the root-soil interface, i.e., the rhizosphere microbiota, under limiting and sufficient nitrogen supplies can be a key step towards economically and environmentally sustainable crop production [7].

This is becoming an urgent research priority for crop wild relatives, which unlike domesticated varieties, have not been selected to respond to mineral fertilisers and typically have evolved under soil stress conditions, such as nutrient deficiency. It is

hypothesized that to thrive in these conditions, crop wild relative engage in mutualistic interactions with soil microbes and, consequently, their microbiota may represent an untapped resource of plant-probiotic functions [8].

Do wild and domesticated plants assemble a distinct microbiota under sufficient and limiting nitrogen supplies? And, if so, what microbes and metabolic functions are influenced by nitrogen applications? Furthermore, do these responses differ in the rhizospheres of wild and domesticated plants? Addressing these questions will be key towards the development of plant varieties capable of recruiting a beneficial microbiota for optimum plant growth under reduced nitrogen applications.

Barley (*Hordeum vulgare*) represents an attractive model to investigate plant-microbiota interactions in crop plants. It is one of the first domesticated crops which today is the fourth most cultivated cereal worldwide [9]. At the same time it is an experimentally-tractable organism: the barley genome has been sequenced [10] and cultivated and wild barley genotypes are readily available to study the impact of crop domestication on plant traits [11]. We previously demonstrated that domesticated (*H. vulgare* ssp. *vulgare*) and wild (*H.vulgare* ssp. *spontaneum*) barley genotypes host contrasting microbiotas [12] and that perturbation of an individual root trait impacts on the recruitment of individual members of the rhizosphere bacterial communities [13]. These data unequivocally point at the barley genotype as a determinant of the microbial communities thriving at the root-soil interface.

Here we tested the hypothesis that the host genotype-mediated selection of the rhizosphere microbiota is modulated by, and responds to, nitrogen application in soil. We compared the plant physiological responses of two wild and a modern (domesticated) barley genotypes grown under controlled conditions in soils amended with and without mineral nitrogen. These data were integrated with a comprehensive characterisation of the rhizosphere microbiota using a combined Illumina 16S rRNA gene amplicon and shotgun sequencing approaches. Finally, a plant-soil feedback experiment was performed to determine how distinct structural and functional configurations of the barley microbiota impact on plant growth.

Results

Fertiliser application impacts on barley growth, nitrogen uptake and soil nitrogen availability

We selected an elite variety, the cultivar “Morex” (referred to as ‘Modern’ in the text), which is a reference for genetic [10] and metagenomics [12] investigations in this

131 species. The wild barley genotypes B1K-31-01 and B1K-12-10, hereafter 'North' and
132 'Desert', respectively, were selected from the ecologically referenced wild barley
133 accessions from the Israeli geographic area and belonging to the 'B1K' collection [14].
134 These genotypes were selected for their different degree of phenotypic and genotypic
135 relatedness with domesticated genotypes, with the 'North' being more closely related to
136 cultivated barley than the 'Desert' [15].

137 Plants were grown under glasshouse conditions (Methods) in an agricultural soil
138 previously used for microbiota investigation and designated 'Quarryfield' (Additional file 1:
139 Table S1; [13]). We subjected plants to three different treatments: we derived either a full
140 (hereafter N100%) or a quarter (N25%) ammonium nitrate (NH_4NO_3) application from a
141 modified Hoagland's solution [16] as well as a treatment with no nitrogen fertiliser (N0%).
142 All the other plant macro and micronutrients were provided at an optimum level in the three
143 treatments to ensure that barley growth would not have been limited by any mineral other
144 than nitrogen (Additional file 1: Table S2). At early stem elongation we sampled plant
145 specimens and determined aboveground plant biomass, plant nitrogen content as well as
146 concentrations of $NH_4^+$and NO_3^- in rhizosphere and unplanted soil (Additional file 2:
147 Supplementary worksheet ws1).

148 Plant growth of Modern and Desert genotypes was not significantly impacted by N
149 application as opposed to the North genotype, whose aboveground biomass at N0% was
150 significantly lower than the other genotypes ($p < 0.05$, Kruskal–Wallis non-parametric
151 analysis of variance followed by Dunn's post hoc test; Figure 1). Conversely, the dynamic
152 of plant N displayed a more pronounced gradient: at N100% plants accumulated
153 significantly more nitrogen than plants maintained at N0%, while plants maintained at
154 N25% displayed an intermediate phenotype.

155 These results mirrored those of the residual concentrations of NH_4^+ and NO_3^- in soil
156 at the time of sampling, in both unplanted and planted pots. Specifically, soil maintained at
157 N100% displayed significantly higher concentrations of both NH_4^+ and NO_3^- compared to
158 the N0% treatment and intermediate values for the N25% treatment ($p < 0.05$, Kruskal–
159 Wallis non-parametric analysis of variance followed by Dunn's post hoc test; Additional file
160 1, Figures S2 and S3).

161 These experiments confirmed that the Quarryfield soil is indeed limited in Nitrogen
162 for plant uptake and the treatments adopted created a gradient of this mineral in both the

unplanted soil and in the rhizosphere specimens. This motivated us to investigate how this gradient impacted on microbiota taxonomic and functional composition.

Taxonomic diversification of the barley microbiota under different nitrogen treatments

To study the impact of N application on the composition of the barley microbiota, we firstly determined the taxonomic affiliation of the 8,874 Operational Taxonomic Units (OTUs) identified in our 16S rRNA gene survey (Additional file 2: Supplementary worksheets 2 and 3). This approach revealed that rhizosphere profiles are dominated by members of the phyla Actinobacteria, Bacteroidetes and Proteobacteria irrespective of the treatment and the genotype (Figure 2). To gain further insights into the relationships between N application and microbial recruitment at the root-soil interface, we first filtered in silico low-abundance and poorly reproducible OTUs (Methods). This allowed us to retain a total 2,628 OTUs and 4,868,770 high quality sequencing reads, representing over 95% of the initial high-quality sequencing reads. First we inspected the within sample diversity which is alpha diversity. Despite a general trend of decreasing richness and evenness directly related to N-application, there was no significant difference between rhizosphere samples (Kruskal–Wallis test at $p > 0.05$; Additional file 1: Figure S4). Next, we investigated between-sample diversity, which is betadiversity. Strikingly, both Bray-Curtis and Weighted unifrac distances were capable of differentiating soil from rhizosphere profiles as evidenced by a segregation of either class of samples in ordination plots (Figure 3). Consistently, we identified significant effects on the microbial communities for genotype and treatment, whereas their interaction was not significant (Permanova $p<0.01$, 5,000 permutations, Table 1). This was further manifested by a graphical separation of the N treatments in the rhizosphere samples and a less marked genotype effect, especially at N0%. While the genotype effect was almost equal in the two dissimilarity metrics and explained ~15% of the variance, the treatment effect resulted in a higher Bray-Curtis metric (~24%) compared with the proportion explained by the weighted unifrac (18% Permanova $p<0.01$, 5,000 permutations, Table 1).

This indicates that the N-treatment significantly impacted on the plant-mediated recruitment cues of the rhizosphere microbiota and predominately on the abundance of its members rather than on their phylogenetic relatedness.

To identify individual microbes supporting the observed diversification, we implemented a series of pair-wise comparisons among treatments and genotypes. Interestingly, in Modern and Desert genotypes we observed that the number of OTUs significantly enriched in and discriminating between rhizosphere profiles from unplanted

soil samples was inversely related to the amount of nitrogen applied (Figure 4, N0% Additional file 2: Supplementary worksheets 4 and 6; N25% Additional file 2: Supplementary worksheets 7 and 9; N100% Additional file 2: Supplementary worksheets 10 and 12; Wald test, $p<0.05$, FDR corrected). Conversely, this trend failed to emerge in the North genotype (Figure 4, Additional file 2: Supplementary worksheets 5-8-11; Wald test, $p<0.05$, FDR corrected). When we inspected the differential recruitment pattern between genotypes, we noticed that, regardless of the comparison performed, plants not amended with further nitrogen (i.e., N0%) assembled a more distinctive, genotype-specific, microbiota compared to plants exposed to N100%(Figure 4, N0% Additional file 2: Supplementary worksheets 13-15; N25% Additional file 2: Supplementary worksheets 16-18; N100% Additional file 2: Supplementary worksheets 19-21; Wald test, $p<0.05$, FDR corrected). Of note, these genotype-specific signatures on the rhizosphere communities were characterised by distinctive taxonomic profiles, with a bias for members of the phyla Bacteroidetes, Firmicutes, Proteobacteria and Verrucomicrobia (Additional file 1, Figures S5).

Owing to the fact that residual nitrogen in soil at the time of sampling significantly increases from N0% to N100% (Additional file 1: Figure S2 and S3), our observations indicated that nitrogen availability for plant uptake is a factor *a*) modulating the microhabitat- and genotype-dependent recruitment cues of the barley microbiota, by *b*) promoting the selective enrichment of individual taxa in the rhizosphere and *c*) whose magnitude is maximised when no further nitrogen is applied to the system.

Modern and Desert microbiotas display distinct functional configurations under low N conditions

To gain insights into the functional significance of the differential bacterial recruitment under N-limiting conditions, we implemented a shot-gun metagenomics approach. We generated over 412M paired-end sequencing reads from 12 bulk soil and rhizosphere samples (Additional file 3: Supplementary worksheet 1). Upon in-silico removal of low quality sequences we were able to classify 253,519,694 sequencing reads (mean per sample=21,126,641, max= 26,643,619 min= 16,065,595, representing ~61% of the initial reads) against the RefSeq database and we identified 61 Prokaryotic and Eukaryotic phyla as well as other unclassified reads, including virus-derived (Additional file 3: Supplementary worksheet 2). Bacteria emerged as the dominant members of both bulk soil and rhizosphere profiles: more than 96% of the annotated sequences were classified as bacteria (Additional file 1: Supplementary figure S6). Closer inspection of the sequencing data further revealed that 9 bacterial phyla, namely Acidobacteria,

Actinobacteria Bacteroidetes, Cyanobacteria, Chloroflexi Firmicutes, Planctomycetes, Proteobacteria and Verrucomicrobia, cumulatively accrued more than 92% of the classified reads across samples (Additional file 3: Supplementary worksheet 2). Despite numerical differences between the sequencing approaches we adopted, and with the notable exception of members of the phylum Cyanobacteria, the relative abundances of these dominant taxa retrieved with the shotgun metagenomics significantly correlate with those obtained with the amplicon sequencing survey from samples exposed to N0% (Pearson product-moment correlation r=0.81, $p < 0.05$; Figure 5).

These observations suggest that, in the tested conditions, the barley microbiota is *a*) dominated by bacteria and *b*) the reconstruction of taxonomic composition at higher rank appeared minimally perturbed by the sequencing approach adopted.

Next, we investigated the metabolic capacities of the bulk soil and rhizosphere communities by analysing their functional annotation against the SEED database. This approach allowed us to retrieve 28 'level-1' subsystems (Additional file 3: Supplementary worksheet 3). Closer inspection of the data revealed a significant impact of both the microhabitat and, within rhizosphere samples, the barley genotype on the relative abundances of these functions (Additional file 1: Figure S7; Permanova $p<0.05$, 5,000 permutations). In particular, we identified 9 functions underpinning a 'rhizosphere effect' on the microbiota, owing to the fact these functions were enriched in rhizosphere specimens compared to unplanted soil controls (Additional file 3: Supplementary worksheet 4, Welch t-test, $p<0.05$, FDR corrected, Table 2). Similarly, we identify significant host genotype-specific signatures in the metabolic potential of these communities. In particular, the microbiota of the Modern genotype enriched for function encoding for RNA metabolism as well as cell wall and capsule (Additional file 3: Supplementary worksheet 5, ANOVA $p <0.05$, FDR corrected, followed by Tukey-Kramer post hoc test; Figure 6). Conversely, the microbiota of the Desert genotype displayed an increase of the relative abundance of sequences encoding for sulphur and, notably, nitrogen metabolism (Additional file 3: Supplementary worksheet 5, ANOVA $p <0.05$, FDR corrected, followed by Tukey-Kramer post hoc test; Figure 6).

Taken together, our data indicate that, under conditions limiting plant N uptake, wild and modern plants assembled distinct functional configurations of the microbiota which differentiated between unplanted soil profile and plant-associated communities and, among these latter, between individual barley genotypes.

Genome reconstruction of the bacteria thriving at the barley root-soil interface

As a first step towards a further characterisation of the functional potential of the microbiota colonising the rhizosphere of wild and modern genotypes, we attempted reconstructing genomes of individual members of the barley microbiota. We assembled the generated metagenomics reads and combined contigs with similar nucleotide composition and differential abundance across samples. This resulted in the reconstruction of 62 Metagenome-Assembled Genomes (MAGs) with a completion of > 60 % according to the presence of a minimal set of essential genes (Methods). Of the 62 reconstructed MAGs, 28 displayed a contamination rate below 5% and were selected for further characterisation (Figure 7).

These high-quality MAGs displayed an average reconstructed size of 4.35Mb and a bias for Bacteroidetes and Proteobacteria. In particular, nearly half of the reconstructed 'high-quality' MAGs (13 out of 28) were classified as Sphingobacteriales and Flavobacteriales. In addition to that, we retrieved 3 genomes each for Burkholderiales and Xanthomonadales, two members each for Pseudomonadales and Sphingomonadales, a member of Rhizobiales and four other genomes that could not be resolved at order level (Additional file 4: Supplementary worksheet 1).

An active Desert microbiota is required for optimum barley growth in Quarryfield soil

To establish a causal relationship between structural and functional configurations of the rhizosphere microbiota and plant growth, we performed a plant-soil feedback experiment by growing a modern variety into soils previously used for the growth of either a modern or a Desert genotypes amended with a N0% solution. At the end of the experiment, plants grown in the 'Desert genotype soil' displayed significant growth differences when exposed to either native or heat-inactivated soils, while these differences were not significant for plants maintained in 'Modern genotype soil' (Figure 8, two-way ANOVA followed by TukeyHSD test, $p < 0.05$, Additional file 5: Supplementary worksheet 1). When we inspected 19 chemical and physical parameters characterising the conditioned soils used, we failed to single out a 'Desert-specific' parameter. Rather, a limited number of properties, namely phosphorus, ammonium, manganese, sulphur, sodium, iron, copper, zinc concentrations as well as pH, explained the majority of the variance among samples and differentiated between native soil and their heat-treated counterparts, irrespective of the initial genotype used (Figure 8, statistical values for the individual properties: $p < 0.01$, $R2 > 0.8$; 5,000 permutations; Additional file 5: Supplementary worksheets 2 and 3). Thus, the chemical and physical perturbations produced by the heat treatment alone cannot explain the 'Desert-specific effect' observed

in plants grown in tested conditions. Although we cannot exclude that other or additional chemicals produced by either genotypes (e.g., allelochemical compounds [17]) influenced, at least in part, the observed plant phenotype, our experiment suggest that an active wild barley microbiota is required for optimum barley growth in the tested conditions.

Discussion

This study provides novel insights into the dynamics of the composition and metabolic capabilities of the rhizosphere microbiota of distinct barley genotypes grown under controlled conditions in an agricultural soil and contrasting N treatments.

Regardless of the treatment applied, we identified a 'rhizosphere effect' on the composition of the barley microbiota: this effect refers to a selective bacterial enrichment and is congruent with earlier studies conducted in barley [12, 13] and other cereal crops such as maize [18, 19] and rice [20]. What emerged from our investigation is the nitrogen-dependency of this process: the more this mineral is applied through fertilisers, the less plants tend to recruit a diverse microbiota. Likewise, the magnitude of the 'genotype effect', was maximal at N0%, when measurements of residual N in the rhizosphere were 0 mg Kg^{-1}, while it was nearly "obliterated" at N100%, when concentrations of residual NO_3^- exceeded 200 mg Kg^{-1} (compare the number of differentially enriched OTUs between genotypes at N0%, N25% and N100% in Figure 4).

This is reminiscent of the observation that in *Medicago truncatula*, a model legume plant, nitrogen availability impacted, in a plant genotype-dependent manner, the bacterial microbiota recruited at the root-soil interface [21]. Although the modulation of the rhizosphere microbiota in legumes has been associated to plant genes implicated in the establishment of symbiosis with N_2-fixing bacteria rather than nitrogen nutritional status per se [22], it is conceivable that this latter modulates, at least in part, host-microbe interactions at the root-soil interface.

For example, nitrogen availability drives the quality and the quantity of plant rhizodeposits: under hydroponic conditions, nitrogen-starved maize plants exude significantly less Aspartate, Tyrosine, Isoleucine and Lysine as well as Maltose compared to plants maintained under optimal nutritional conditions [23]. In turn, these metabolic changes can impact on microbial activity in the rhizosphere [24]. Although additional experiments are required to untangle the molecular mechanisms linking N-availability with microbiota diversification in barley, evidence of this link has been gathered also in other members of the family *Poaceae*. For instance, the addition of nitrogen fertilisers to soil-

grown perennial ryegrass (*Lolium perenne*), a species which diverged from barley ~22-30 million years ago [25], reduces the, microbial-mediated, mineralisation of soil organic matter in planted soils but not in unplanted soil controls [26]. Similarly, results from a predictive metagenomics investigation conducted in wheat revealed that the exposure to either organic or mineral fertiliser triggers a differential nitrogen metabolism in the rhizosphere of the tested plants [27].

The genotype-dependent diversification of the barley microbiota at N0% revealed that members of the phylum Verrucomicrobia, specifically the orders Pedosphaerales and Opitutales, among the bacteria preferentially recruited by Desert genotypes (Additional file 1: Figure S5; Additional file 2: Supplementary worksheets 25-26). Although members of this phylum have not consistently been identified among rhizosphere competent bacteria [28], recent investigations conducted in maize suggest that members of the orders Pedosphaerales and Opitutales can indeed respond to host genotype-dependent features [19]. Conversely, the North genotype promotes the selective enrichment of members of the phylum Firmicutes, order Bacillales (specifically genus Paenibacillus; Additional file 2; Supplementary worksheets 27-28) which recently were identified as a 'keystone taxa' in the common bean rhizosphere microbiota [29]. The fact that we analysed only one soil and a limited number of plant genotypes makes it difficult to infer first principles, however it is tempting to speculate that nitrogen availability is a determinant of plant-associated microbial communities capable of selectively overwriting "canonical" recruitment cues of the rhizosphere microbiota.

These observations motivated us to further investigate the functional potential of the rhizosphere microbiota under conditions limiting plant nitrogen uptake. Our shot-gun sequencing approach revealed that these communities are largely dominated by prokaryotes: more than 96% of the annotated sequences were classified as bacteria which is strikingly similar to a previous investigation conducted in a soil with different physical and chemical characteristics [12]. This suggests that bacteria are efficient coloniser of the barley rhizosphere irrespective of the soil type. Although the dominance of bacterial sequences over other members of the microbiota is not unusual in soil metagenomes [30] and differences of a least an order of magnitude between rRNA genes copy numbers between bacteria and fungi have previously been reported from soil [31], it is important to metion that both the protocol used for microbial DNA preparation and the databases used for sequencing annotation [32] can 'artificially inflate' the proportion of bacteria among the analysed sequences.

Regardless of the potential biases introduced by the selected protocols, the congruence between amplicon and shotgun sequencing taxonomic profiles prompted us to reconstruct the functional potential of the barley microbiota using a two-pronged approach. Shot-gun metagenomics revealed that distinctive traits of these communities include the enrichment of functions implicated in host-microbe interactions as well as mineral metabolism, which were previously catalogued as traits underpinning 'rhizosphere competence' [28]. In particular, we identified motility and chemotaxis as well as Iron acquisition & metabolism as the functions displaying the more marked enrichment. Interestingly, these enrichments are congruent with previous metagenomics study conducted in barley, but in a different soil type [12] , and in other crops [33].

In parallel we found evidence of genotype-dependent signatures in the functional potential of the barley microbiota. For instance, the Desert genotype enriches for functions implicated in mineral metabolism, namely nitrogen and sulphur, when compared with unplanted soil controls and the other two genotypes. The fate of these two essential plant micronutrients in the rhizosphere is tightly linked: nitrogen and sulphur are essential constituents of amino acids and proteins and their uptake and metabolism in plant is co-regulated [34]. For example, sulphur-deprived, soil-grown barley plants accumulate more nitrate in their shoots than their cognate controls not exposed to such a nutritional starvation, suggesting that sulphur deficiency directly impacts on nitrogen metabolism in planta [35]. Thus, the dual enrichment of nitrogen and sulphur metabolisms in the Desert rhizosphere may underpin either a mutual relationship, whereby rhizosphere microbes facilitate the release of a "balanced" set of minerals from organic substrate for plant nutrition [36] or a host-microbe competition for these resources.

Conversely, the Modern varieties were characterised by an enrichment of functions implicated in RNA metabolism as well as cell wall and capsule. While these latter functions are reminiscent of a defensive strategy against antimicrobial compounds [37, 38], an enhanced RNA metabolism in the Modern rhizosphere may be directly implicated in nitrogen metabolism. For instance, a comparative metagenomics investigation of agriculturally managed soil subjected to long term N-fertilisation revealed a direct relationships between these function and N application [30] . The observation that these functions are significantly enriched in the rhizosphere of 'Modern' at N0% may suggest that exudates of cultivated varieties are a "richer" source of N compounds compared to the wild barley. Evidence supporting this scenario could be derived from the fact that the

incubation of a substrate with high C/N ratio such as lignin triggers more microbial respiration in the rhizosphere soil of a modern variety compared to a wild ancestor [39].

Our observations suggest a multi-step assembly process shaping the metabolic potential of the barley rhizosphere microbiota. The functions enriched by rhizosphere bacteria appear required for the establishment of a microbial community at the barley root-soil interface. These microbial assemblages are further fine-tuned by a host- and soil-dependent selection operating at the level of the individual genotypes and mediating their adaptation to the environment. This scenario is expressly similar to the multi-step assembly process proposed for the taxonomic diversification of the microbiota thriving at the root-soil interface [20, 28].

A prediction of this observation is the existence of a molecular link between structure and functions of the barley microbiota. Although our genome reconstruction exercise prevented us ascertaining OTUs/MAGs relationships, due to the multi-copy nature of rrn operons, and to perform a genotype-specific functional enrichment, due to the differential coverage across samples, it represented a key step towards the resolution of this link. Similar to a recent investigation which led to the identification of a 'plant-protecting' bacterium in tomato rhizosphere [40], the reconstructed genomes will facilitate the identification of putative rhizosphere- and barley-competent bacteria among existing, genome-annotated, microbial collections (e.g., [41]) for recolonization experiments.

Our plant-soil feedback experiment revealed a limited, but significant, effect of the wild barley microbiota for plant growth. Closer inspection of the physical and chemical characteristics of the soil substrates provided insights into the mechanisms underpinning this effect on plant growth. For instance, ammonium and sulphur were significantly more abundant in heat-treated samples compared to 'native' soils. Studies conducted with a modern barley variety revealed that an increased level of sulphate in the rhizosphere inhibits the rhizospheric arylsulfates activity [42], i.e., the enzyme catalysing the hydrolysis of sulphate esters into mineral forms available for plant uptake [43]. Conversely, a microbiota better adapted to nitrogen and sulphur metabolism, such as the one associated with the Desert genotype, may offset this mineral imbalance contributing to a more efficient plant sulphur uptake in the barley rhizosphere and, at least in part, to the observed growth phenotype.

Our results concur with previous studies conducted with model plants [44] and a cereal crop [45] which revealed a microbiota-mediated impact on traits, such as flowering

time and pest resistance, of recipient plants in plant-soil feedback experiments. Our results, coupled with the observation that metabolic capacities of the microbiota are, at least in part, genetically inherited in experimental population between modern and wild barley genotypes [39], may set the stage for the deployment of functions of the microbiota for plant breeding purposes. This may have an immediate impact on sustainable crop production: decades of selection for barley cultivars capable of responding to fertilisers have not yet achieved the maximum nitrogen uptake efficiency for this species [46]. Our results suggest that this "gap" can be filled, at least in part, by more efficient interactions with the soil biota.

Conclusions

Our results point at nitrogen availability for plant uptake as a driver capable of modulating the host control of the rhizosphere microbiota. This modulation is exerted at taxonomic level and underpins a functional diversification of the microbiota. Each genotype retains a specific functional signature and a wild barley from Desert area appears to preferentially recruit functions implicated in nitrogen and sulphur metabolism. Our plant-soil feedback experiments suggest that functions enriched by the wild barley microbiota have a limited, but significant, positive impact on barley growth. Our results constitute fundamentally novel insights into the adaptation of crop plants to limited nitrogen applications and will set the stage to exploit plant-microbiota interactions for sustainable management of nitrogen in agroecosystems.

Methods

Soil

The soil was sampled from the agricultural research fields of the James Hutton Institute, Invergowrie, Scotland, UK in the Quarryfield site (56°27'5"N 3°4'29"W). This field was left unplanted and unfertilised in the three years preceding the investigations and previously used for barley-microbiota interactions investigations [13]. The chemical and physical characteristics of the soil are presented in Additional file 1: Table S1.

Plant growth conditions

Barley seeds were surface sterilized as previously reported [47] and germinated on 0.5% agar plates. Seedlings displaying comparable rootlet development were sown individually in 12-cm diameter pots containing approximately 500g of the 'Quarryfield' soil, plus unplanted pots filled with bulk soil as controls. Plantlets one week old were transferred for two weeks to a growth room at 4 °C for vernalisation. After this initial

treatment, plants were grown in a randomized design in a glasshouse at 18/14 °C (day/night) temperature regime with 16 h day length (Additional file 1: Figure S1).

Nitrogen treatments

The nutrient solution applied in this study, i.e. N100%, N25% and N0% are reported in Additional file 1: Table S2. The nutrient solution was applied with watering consisting of a weekly input of 25ml of the nutrient solution per kg of soil. The application started two days after planting, was interrupted during the vernalisation and relapsed once the plants were transferred to the growing glasshouse and they reach early stem elongation. Fourteen treatments were applied with a total of 312.50 mg (NO_3^-); 81.4 mg (NH_4^+) for the N100% solution, 78.13 mg (NO_3^-); 20.04 mg (NH_4^+) for the N25% solution, and 0 mg of (NO_3^- and NH_4^+) for the N0% solution per pot.

Bulk soil and rhizosphere DNA preparation

We organised a randomised design with two factors, sample type and nitrogen treatment, having four (unplanted soil, Desert, North and Modern) and three (N100%, N25% and N0%) levels, respectively and 5 replications per combinations of levels Samples were maintained under controlled conditions (see Plant Growth Conditions) and upon cultivation of the specimens and DNA preparation we were able to retain at least 3 biological replicates/sample type/treatment alongside wo unplanted soil control pots inoculated with water agar plugs (Additional file 2: Supplementary worksheet 1). At early stem elongation, after a 5-week growth period in glasshouse (plus the previous 2 weeks of vernalisation) plants were excavated from the soil and the stems were separated from the roots. The uppermost 6 cm of the root system were detached from the rest of the root corpus and processed for further analysis. Stems were oven dried at 70°C for 48 hours and the dry weight recorded. The roots were shaken manually to remove excess of loosely attached soil. For each barley plant, the top 6cm of the seminal root system and the attached soil layer was collected and placed in sterile 50ml falcon tube containing 15ml phosphate-buffered saline solution (PBS). Rhizosphere was operationally defined, for these experiments, as the soil attached to this part of the roots and extracted through this procedure. The samples were then vortexed for 30 seconds and aseptically transferred to a second 50ml falcon containing 15ml PBS and vortexed again to ensure the dislodging and suspension of the rhizosphere. Then, the two falcon tubes with the rhizosphere suspension were mixed and centrifuged at 1,500g for 20 minutes to precipitate the rhizosphere soil into a pellet, flash frozen with liquid nitrogen and stored at -80°C, until further analysis.

In addition, we incubated water agar plugs (~1 cm^3) into two unplanted soil pots and we maintained them as control samples among the experimental pots to monitor the effect of this medium on the soil microbial communities.

DNA was extracted from unplanted soil and rhizosphere samples using FastDNA™ SPIN Kit for Soil (MP Biomedicals, Solon, USA) according to the manufacturer's recommendations and stored at -20°C.

Plant and Soil Nitrogen determination

To assess the N content of the plant, at the time of sampling a newly expended leaf was sectioned from every plant, freeze-dried, ball milled, and N content measured in an Elemental Analyser CE-440 (Exeter Analytical Inc, UK)

The soil content of the pots was sieved through a 2mm mesh sieve. Five grams of soil was added with 25mL of 1M KCl and the resulting solution mixed in a tube rollers for 1hour at ~150 rpm. Supernatant was transferred to 50mL falcon tubes and centrifuged for 15min at 5,000 rpm, then the supernatant was subject to another round of centrifugation. The supernatant was transferred to a falcon tube and analysed in the Discrete Analyser Konelab Aqua 20 (Thermo Fisher, Waltham, USA) in the analytical services of The James Hutton Institute (Aberdeen, UK). In parallel, ~10 g from the sieved soil was oven dried at 70 C for 48h and dry weight recorded to express the analytical results in NO_3^- and NH_4^+ in mg/kg of soil.

Preparation of 16 rRNA gene amplicon pools

The hypervariable V4 region of the small subunit rRNA gene was the target of amplification using the PCR primer pair 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The PCR primers had incorporated an Illumina flow cell adapter at their 5' end and the reverse primers contained 12bp unique 'barcode' for simultaneous sequencing of several samples [48]. PCR reactions, including No-Template Controls (NTCs) for each barcoded primer, were performed as previously reported with the exception of the BSA concentration at 10mg/ml per reaction [13]. Only samples whose NTCs yielded an undetectable PCR amplification were retained for further analysis.

Illumina 16S rRNA gene amplicon sequencing

The pooled amplicon library was submitted to the Genome Technology group, The James Hutton Institute (Invergowrie, UK) for quality control, processing and sequencing. Amplicon libraries were amended with 15% of a 4pM phiX solution. The resulting high-

quality libraries were run at 10 pM final concentration on an Illumina MiSeq system with paired-end 2x 150 bp reads [48] to generate the sequencing output, the FASTQ files.

Sequencing reads processing

Sequencing reads were processed and analysed using a custom bioinformatics pipeline. First, QIIME (Quantitative Insights into Microbial Ecology) software, version 1.9.0, was used to process the FASTQ files following default parameters for each step [49]. The forward and reverse read files from individual libraries were decompressed and merged using the command join_paired_ends.py, with a minimum overlap of 5bp between reads. Then, the reads were demultiplexed according to the barcode sequences and joined by the overlapping paired-end (PE). Quality filtering was performed using the command split_libraries_fastq.py, imposing a minimum acceptable PHRED score '-q' of 21. Next, these high quality reads were truncated at the 250th nucleotide using the function 'fastq_filter' implemented in USEARCH [50]. Only these high-quality PE, length-truncated reads were used for clustering in Operational Taxonomic Units (OTUs) a 97% sequence identity. OTUs were identified using the 'closed reference' approach against the chimera-checked Greengenes database (version 13_5) [51]. OTU-picking was performed using the SortMeRNA algorithm [52], producing in an OTU table containing the abundance of OTUs per sample plus a phylogenetic tree. Singleton OTUs, (OTUs accounting for only one sequencing read in the whole dataset) and OTUs assigned to chloroplast and mitochondria (taken as plant derived sequences) were removed. Taxonomy matrices, reporting the number of reads assigned to individual phyla, were generated using the command summarize_taxa.py. The OTU table, the phylogenetic tree and the taxonomy matrix, were further used in R for visualizations and statistical analysis.

Illumina shot-gun metagenomics: sequencing generation and annotation

We generated a new set of 3 bulk soil and 3 rhizosphere DNA preparations from each of the three genotypes tested (i.e., 'Desert', 'North' and 'Modern') from specimens maintained in Quarryfield soil under N0% conditions as described above. These 12 new preparations were quantified and submitted to the LGC Genomics sequencing service (Berlin, Germany) where they were used to generate DNA shotgun libraries using the Ovation Rapid DR Multiplex System 1-96 NuGEN (Leek, The Netherlands) kit following manufacturer's recommendations. These libraries were the run simultaneously into an individual Illumina NextSeq500 run following manufacturer's recommendations with the 2 X150bp chemistry and generated a total of 412,385,413 read pairs. After sequencing read pairs were de-multiplexed according to the samples barcodes using the Illumina bcl2fastq

2.17.1.14 software. FASTQ files corresponding to forward and reverse reads from each sample were submitted to the analytical pipeline of MG-RAST for merging, dereplication, screening against host contamination and dynamic trimming using the default parameters, with the exception of host contamination which was performed against the A. thaliana TAIR9 reference sequences. We use MG-RAST also to annotate these high-quality metagenomics reads against the RefSeq database (taxonomy information, Phylum level) and the Subsystem (functional annotation, Level 1). In both cases we used the MG-RAST's default parameters (i.e., e-value -5; % Identity 60%; length 15) and impose an abundance threshold of at least 10 reads for a future, either a taxon or a functional category, to be retained in the resulting annotated tables. The Subsystem-annotated table was exported as tab-delimited file, converted in BIOM format and rarefied at an even sequencing depth using the functions 'biom convert' and single_rarefaction.py in QIIME, respectively.

Metagenome-Assembled Genomes Reconstruction

Microbial genome binning was performed following the approach described [53]. Briefly, 12 individual assemblies were executed with IDBA_UD [54]. All assemblies were combined in a single meta-assembly file. Reads were then mapped to the meta-assembly using Bowtie2 [55]. Mapped reads and the meta-assembly file were used for the initial binning step with the software Metabat v.0.32.4 [56]. Bin completeness, contamination and strain heterogeneity were performed with CheckM v.1.0.4 [57]. The contamination index indicates the percentage of specific marker genes that are found in multiple copy number, and strain heterogeneity values indicate the percentage of multi-copy marker genes with more than a 90% sequence identity. Coverage was calculated on the basis of the mapped reads. It was computed with the formula C = LN/B where L is read length in bp, N is the number of reads mapping and B is the total length of the assembled genome. In total, 229 metagenomic-assembled genomes (MAGs) were recovered from this initial binning step. Sixty-two MAGs with a minimum completeness of 60% were then selected for further analysis. To gain an insight on the phylogenetic affiliation of each MAG, three different approaches were followed. First, a BLASTn search of the first 5000 nt of the 10 largest contigs MAGs was performed against the NCBI database. Then, the phylogenetic profile of each MAG was estimated with CLARK; a k-mer based classifier. Finally, a Composition Vector Tree (CVTree [58]) was constructed using the proteome of each recovered MAG.

Plant-soil feedback experiment

We grew Desert and Modern genotypes in 'Quarryfield' soil supplemented with a N0% nutrient solution under controlled conditions (see Plant growth conditions). We selected these two genotype since, in the tested soils, they host a taxonomic and functional distinct microbiota. At early stem elongation we removed the plants from the soil and we harvested the residual soil and kept it separated in a genotype-wise manner. We reasoned that at the end of cultivation the soils would have been enriched for the specific taxa/functions associated to either genotypes This residual soil, either in a 'native form', i.e., not further treated after sampling, or after being exposed to a heat-treatment (126°C for 1 hour, repeated twice at an interval of ~12 hours), was used as a substrate for a subsequent cultivation of a recipient Modern barley genotype. These plants maintained under controlled conditions (see Plant growth conditions) and supplemented with a N25% solution to compensate for the near completed depletion of this mineral in the previous cycle of cultivation (compare the NH_4^+ and NO_3^- concentrations of rhizosphere specimens at N0% and N25% in Additional file 1: Figures S2 and S3). At early stem elongation plants were harvested at their aboveground biomass determined after incubating stems and leaves at 70°C for 48 hours. For each conditions we initially contemplated 5 biological replicates and the entire experiment was performed three times. At the end of each replicated experiment, the residual soil was collected and arranged in a condition-wise manner and subjected to chemical and physical characterisation (Yara United Kingdom Ltd., Grimsby, United Kingdom).

Statistical analyses I: univariate datasets and 16S rRNA gene alpha and betadiversity calculations

Analysis of the data was performed in R software using a custom script with the following packages: Phyloseq [59] for processing, Alpha and Beta-diversity metrics; ggplot2 [60] for data visualisations; Vegan [61] for statistical analysis of beta-diversity; Ape [62] for phylogenetic tree analysis; PMCMR [63] for non-parametric analysis of variance and Agricolae for Tukey post hoc test [64]. For any univariate dataset used (e.g., aboveground biomass) the normality of the data's distribution was checked using Shapiro–Wilk test. For datasets normally distributed, the significance of the imposed comparisons was assessed by an ANOVA test followed by a Tukey post hoc test. Non-parametric analysis of variance were performed by Kruskal-Wallis Rank Sum Test, followed by Dunn's post hoc test with the functions kruskal.test and the posthoc.kruskal.dunn.test, respectively, from the package PMCMR. We used Spearman's rank correlation to

636 determine the similarity between unplanted soil profiles and bulk soil samples amended
637 with water agar plugs (Additional file 1: Table S3).
638
639 For the analysis of microbiota data, first, a 'phyloseq object' was created allowing
640 joint analysis of the mapping file (metadata information), OTU table, phylogenetic tree and
641 the taxonomy matrix. For Alpha-diversity analysis, the OTU table was rarefied (assigning
642 the same number of reads to each sample) and Chao1, Observed OTUs and Shannon
643 indices calculated using the function estimate richness in Phyloseq package. Chao1: that
644 project sequencing saturation, considering rare OTUs; Observed OTUs: that counts
645 unique OTUs in each sample; and Shannon: that measures evenness in terms of the
646 number of OTUs presence and abundance. Beta-diversity was analysed using a
647 normalized OTU table for comparison. For the construction of the normalized OTU table,
648 low abundance OTUs were further filtered removing those not present at least 5 times in
649 20% of the samples, to improve reproducibility. Then, to control for the uneven number of
650 reads per specimen, individual OTU counts in each sample w were divided over the total
651 number of generated reads for that samples and converted in counts per million.
652 Transforming the OTU table in this manner provides a relative abundance of OTUs per
653 sample. Beta-diversity was analysed using two metrics: Bray-Curtis that considers OTUs
654 relative abundance; and Weighted Unifrac that additionally is sensitive to phylogenetic
655 classification [65]. These dissimilarity matrices were visualized using Principal Coordinates
656 Analysis (PCoA) using the ordinate function in the Phyloseq package. Beta-diversity
657 dissimilarity matrices were assessed by Permutational Multivariate Analysis of Variance
658 (Permanova) using Adonis function in Vegan package over 5,000 permutations to
659 calculate effect size and statistical significance. Version of the individual packages used
660 are described in the individual scripts used (see Data Availability below).

661 ### Statistical analyses II: analysis of OTUs differentially enriched among samples

662 The differential analysis of the OTUs relative abundances was performed a)
663 between individual genotypes and bulk soil samples to assess the rhizosphere effect and
664 b) between the rhizosphere samples to assess the genotype effect. The genotype effect
665 was further corrected for a microhabitat effect (i.e., for each genotype, only OTUs enriched
666 against both unplanted soil and at least another barley genotype were retained for further
667 analysis). The analysis was performed using the DESeq2 method [66] consisting in a
668 moderated shrinkage estimation for dispersions and fold changes as an input for a pair-
669 wise Wald test. This method identifies the number of OTUs significantly enriched in pair-
670 wise comparisons with an adjusted p value (False Discovery Rate, FDR $p < 0.05$). This

method was selected since it outperforms others hypothesis-testing approaches when data are not normally distributed and a limited number of individual replicates per condition (i.e., less than 10) are available [67]. DESeq2 was performed in QIIME with the command differential_abundance.py with the OTU table filtered for low abundance OTUs as an input. To control for potential contaminant OTUs amplified during library preparation, we retrieved a list of potential environmental contaminant OTUs previously identified in our laboratory [68] and we used this list to filter the results of the aforementioned OTU-enrichment analysis.

Statistical analyses III: comparison between 16S rRNA gene and shotgun metagenomics dataset and analysis of metagenomics functions differentially enriched among samples

We determined the proportion of phyla accounting for an average 1% relative abundance across samples. We then retrieved the average relative abundance of the phyla identified as dominant with both sequencing approaches in the 16S rRNA gene dataset (Additional file 2: Supplementary worksheet 3). Relative abundance data (%) of both datasets were log2 transformed and plotted in R using the function ggscatter. The transformed data were inspected for the normality of their distribution using the Shapiro–Wilk test and the Pearson product-moment correlation was subsequently calculated using the function cor.test .

Next we created a Phyloseq object using the design file of the experiment as ‘sample_data’ file and the Subsystem-annotated table, Level 1, rarefied at 5.8M reads per sample as an ‘otu_table’ file. Ordinations and permutational analysis of variance were conducted as described above (see Statistical analyses I: univariate datasets and 16S rRNA gene alpha and betadiversity calculations).

We used STAMP [69] to identify annotated functions differentially enriched between genotypes. Briefly, the Subsystem-annotated table, Level 1, rarefied at 5.8M reads per sample was searched for functions whose relative abundance was significantly enriched in either a) one microhabitat (bulk/rhizosphere) using a Welch t-test or b) at least one microhabitat/genotype using an ANOVA at a p value <0.05 (FDR corrected).

Tables and Figure legends

Weighted Unifrac		
	R2	**Pr(>F)**
Genotype	0.148	<0.001
Treatment	0.185	<0.001
Genotype X Treatment	0.098	0.270
Bray-Curtis		
	R2	**Pr(>F)**
Genotype	0.142	<0.001
Treatment	0.243	<0.001
Genotype X Treatment	0.094	0.184

Table 1. Proportion of variance explained by and statistical significance of the indicated factors on the rhizosphere microbiota

Function	Unplanted soil (reads %)		Rhizosphere (reads %)		p value
	Mean	**SD**	**Mean**	**SD**	
Fatty Acids, Lipids, and Isoprenoids	2.600	0.005	2.644	0.025	$2.1 * 10^{-3}$
Cell wall and capsule	3.898	0.047	4.041	0.084	$3.2 * 10^{-2}$
Iron acquisition & metabolism	0.681	0.024	1.201	0.181	$1*10^{-4}$
Membrane transport	3.983	0.045	4.201	0.100	$3.9*10^{-3}$
Miscellaneous	6.575	0.015	6.690	0.059	$1.4*10^{-3}$
Motility & chemotaxis	0.815	0.016	1.112	0.058	$5.2*10^{-6}$
Potassium metabolism	0.667	0.005	0.796	0.032	$1.2 *10^{-5}$
Regulation & cell signalling	1.010	0.005	1.119	0.039	$1.2*10^{-4}$
Stress responses	2.570	0.011	2.667	0.029	$1.9*10^{-4}$

Table 2. Sequencing reads (Mean and Standard Deviations-SD) assigned to the functional categories enriched in and discriminating between rhizosphere and unplanted soil samples (Welch t-test, P values FDR corrected)

Figure 1 Effect of the Nitrogen treatment on plant growth and mineral uptake. Average aboveground (**a**) biomass and (**b**) nitrogen content (% of plant dry weight) at the time of sampling of the indicated barley genotypes exposed to the three nitrogen treatments depicted by N0%; N25% and N100%, respectively. Upper and lower edges of the box plots represent the upper and lower quartiles, respectively. The bold line within the

box denotes the median. Maximum and minimum observed values are represented by the whiskers. Dots denote outlier observations whose value are 3/2 times greater or smaller than the upper or lower quartiles, respectively. Different letters within the individual plots denote statistically significant differences between means by Kruskal-Wallis non parametric analysis of variance followed by Dunn's post-hoc test ($P < 0.05$).

Figure 2 The dominant phyla of the bulk soil and rhizosphere microbiota are conserved across nitrogen treatment. Average relative abundance (% of sequencing reads) of the dominant phyla retrieved from the microbial profiles of indicated samples supplemented with no additional (N0%), a sub-optimal (N25%) or a sufficient (N100%) amount of mineral nitrogen, respectively. Only phyla displaying a minimum average relative abundance of 1% included in the graphical representation.

Figure 3 Genotype and Nitrogen treatment impact on the barley rhizosphere microbiota composition. Principal Coordinates Analysis of the Weighted Unifrac (**a**) or Bray-Curtis (**b**) dissimilarity matrices of the microbial communities retrieved from the indicated genotypes and subjected to differential nitrogen treatment. Individual shape depicts individual biological replicates.

Figure 4 Enrichments of individual bacteria discriminates between bulk soil and rhizosphere and defines host-genotype effects on the barley microbiota. Number of individual 16S rRNA gene OTUs (**a**) significantly enriched in the indicated rhizosphere microbiotas compared with unplanted soil controls and (**b**) whose relative abundances in the rhizosphere microhabitat differentiates between plant genotypes (Wald test, $p < 0.05$, FDR corrected). Abbreviations: D, 'Desert'; N, 'North'; M, 'Modern'.

Figure 5: **The proportion of dominant taxa in the bulk soil and rhizosphere microbiota at N0% is minimally affected by the sequencing approach.** Proportion of sequencing reads (%, log 2 transformed) assigned to the indicated phyla retrieved from samples exposed to N0%. Individual dots depict the average relative abundance value for the indicated phylum in either the 16S rRNA amplicon sequencing survey (x-axis) or the shotgun metagenomics (y-axis).The blue line depicts the regression lines, the grey shadow the confidence interval, respectively. Pearson product-moment correlation calculated on log2 transformed data ($r=0.81$, $p < 0.05$).

Figure 6: **Selective enrichments of microbial functions discriminate between rhizosphere genotypes.** Proportion of sequencing reads (%, average relative abundances) assigned to the categories of functional genes significantly enriched in and

discriminating between A) 'Desert' and the other samples or B) 'Modern' and the other samples. Bars depict standard deviations, *n*=3 replicates for each sample type. Asterisks denotes significant differences (ANOVA followed by Tukey test, * p <0.05, ** p <0.01, ***p <0.001, FDR corrected).

Figure 7: **Composition Vector Tree (CVTree) displaying the phylogenetic placement of the 62 reconstructed Metagenome Assembled Genomes (MAGs).** The CVtree was based on the MAGs proteome. Only MAGs with a completeness higher than 60% were reported. The highlighted tips correspond to MAGs with less than a 10% contamination. The branch pie charts represent MAGs completeness. Node labels were coloured according to taxonomy. Outer bar plots correspond to contamination rate while the external circles represent MAG coverage in all the three different plant genotypes and soil.

Figure 8: **The heat-inactivated microbiotas impact on barley growth in soil and on the chemical and physical properties of the substrate** **a)** Box plots summarising the aboveground biomass of barley in the indicated conditioned soil. Individual red dots depict measurements of individual replicates, the middle lane in the box represents the median of these measurements. Letters denotes significant differences (ANOVA, p<0.05). **b**) Non-metric multidimensional scaling illustrating relationships among the indicated samples on the basis of selected chemical and physical soil properties. The plot depicts the most significant (R2 > 0.83, p <0.001) parameters explaining the ordination, namely the concentration of ammonium (NH_4), Phosphorus (P), Manganese (Mn), Sulphur (S), Sodium (Na), Iron (Fe), Copper (Cu), Zinc (Zn) and the pH. Arrows point at the direction of change while their length is proportional to the correlation between the ordination and the indicated variables.

Data Availability

The sequences generated in the 16S rRNA gene sequencing survey and the raw metagenomics reads reported in this study are deposited in the European Nucleotide Archive (ENA) under the accession number PRJEB30847. Individual metagenomes are retrievable on the MG-RAST server under the IDs mgm4798244.3; mgm4798274.3; mgm4798349.3; mgm4798388.3; mgm4798507.3; mgm4798563.3; mgm4798641.3; mgm4798894.3; mgm4799467.3; mgm4799972.3; mgm4801514.3; mgm4801719.3.

The scripts used to analyse the data and generate the figures of this study are available at https://github.com/BulgarelliD-Lab/Barley-NT

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Authors’ contribution

The study was conceived by RAT and DB with critical inputs from EP and LB. RAT, AMC, SRA and KBC performed the experiments. JM and PH generated the 16S rRNA sequencing reads. GT and MB conceived and performed the genome reconstruction experiment. RAT, AMC, LP and DB analysed the data. All authors critically reviewed and edited the manuscript, and approved its publication.

Additional files

Additional file 1:

Table S1: Chemical and physical characteristic of the ‘Quarryfield’ soil used in this study. **Table S2**: Composition of the nutrient solutions used in this study. The solution was applied with watering of the plants at a rate of 25 ml of the nutrient solution per Kg of soil. *Micronutrients concentration: 6 µM $MnCl_2$, 23 µM H_3BO_3, 1.6 µM $CuSO_4$, 0.6 µM $ZnSO_4$, 1 µM Na_2MoO_4, 1 µM $CoCl_2$. **Table S3:** Spearman’s rank correlations computed between the average relative abundances (phylum level) of the communities retrieved from unplanted soil samples and unplanted soil amended with “water agar plugs”. **Figure S1**:

Barley plants at the time of sampling. Genotypes and N treatments are arranged as rows and columns, respectively, for data recording. Top row 'Modern', middle row 'North' bottom row 'Desert' genotypes. From left to right: first column 100%N treated plants, second column 25%N treated plants, and third column 0% N treated plants. **Figure S2**: NH4 content of the unplanted soils and rhizospheres. Data gathered at early stem elongation in wild barley genotypes ('Desert 'and 'North') and 'Modern' varieties (Morex) at to three Nitrogen fertilization regimes (0, 25 and 100%). Letters denote significant differences at $p<0.05$ in a Kruskal–Wallis non-parametric analysis of variance followed by Dunn's post hoc test. **Figure S3**: NO3 content of the unplanted soils and rhizospheres. Data gathered at early stem elongation in wild barley genotypes ('Desert 'and 'North') and 'Modern' varieties (Morex) at to three Nitrogen fertilization regimes (0, 25 and 100%). Letters denote significant differences at $p<0.05$ in a Kruskal–Wallis non-parametric analysis of variance followed by Dunn's post hoc test. **Figure S4**: Alpha-diversity calculations of Chao1 (A), Observed OTU's (B), and Shannon (C) recorded for wild barley genotypes ('Desert 'and 'North') and 'Modern' varieties (Morex) at three Nitrogen fertilization regimes (0, 25 and 100%) as indicated in the x-axis. Data computed using OTUs clustered at 97% similarity. No significant difference discriminated between rhizosphere samples (Kruskal–Wallis test at $p > 0.05$). **Figure S5**: Order classification of genotype-enriched OTUs at N0%. Number of OTUs differentially recruited between genotypes (Wald test, $p <0.05$, FDR corrected) assigned to the indicated order the color-coded pair-wise comparisons and A) enriched in 'Modern' against the other genotypes and B) enriched in 'Desert' against the other genotypes. **Figure S6**: Bacteria dominate the bulk soil and barley rhizosphere shotgun metagenomes. Proportion of sequencing reads (% of high quality reads) assigned to a given taxonomic group indicated by a specific colour. Each ring depicts the classification of the shogun reads retrieved from Bulk soil, Desert, North and Modern genotype, respectively, maintained at N0%. **Figure S7**: Functional differentiation of the unplanted soil and the barley rhizosphere microbiota. Principal Coordinates Analysis constructed using a Bray-Curtis dissimilarity matrix computed on the relative abundances (sequencing reads ‰) of the identified SEED1 function. Different shapes denote different microhabitats and genotypes are defined by individual colours.

Additional file 2:

Supplementary worksheet 1: Experimental design and sample description for the 16S rRNA gene sequencing experiment. **Supplementary worksheet 2**: OTUs and individual counts identified the amplicon sequencing survey. **Supplementary worksheet 3**: Matrix depicting the phylum relative abundances of the microbes identified in the amplicon

sequencing survey. **Supplementary worksheets 4-6**: Statistical and taxonomic information of the OTUs differentially recruited between Desert and Bulk, North and Bulk and Modern and Bulk, respectively, at N0%. **Supplementary worksheets 7-9**: Statistical and taxonomic information of the OTUs differentially recruited between Desert and Bulk, North and Bulk and Modern and Bulk, respectively, at N25%.**Supplementary worksheets 10-12**: Statistical and taxonomic information of the OTUs differentially recruited between Desert and Bulk, North and Bulk and Modern and Bulk, respectively, at N100%. **Supplementary worksheets 13-15**: Statistical and taxonomic information of the OTUs differentially recruited between Desert and Modern, North and Desert and Modern and North, respectively, at N0%. **Supplementary worksheets 16-18**: Statistical and taxonomic information of the OTUs differentially recruited between Desert and Modern, North and Desert and Modern and North, respectively, at N25%. **Supplementary worksheets 19-21**: Statistical and taxonomic information of the OTUs differentially recruited between Desert and Modern, North and Desert and Modern and North, respectively, at N100%. **Supplementary worksheet 22**: List of potential contaminant OTUs. **Supplementary worksheets 23-24**: Statistical and taxonomic information of the OTUs enriched in Modern versus North and Desert genotypes at N0%, upon filtering for potential contaminant OTUs. **Supplementary worksheets 25-26**: Statistical and taxonomic information of the OTUs enriched in Desert versus Modern and North genotypes at N0%, upon filtering for potential contaminant OTUs. **Supplementary worksheets 27-28**: Statistical and taxonomic information of the OTUs enriched in North versus Modern and Desert genotypes at N0%, upon filtering for potential contaminant OTUs.

Additional file 3:

Supplementary worksheet 1: Experimental design and sample description for the shotgun experiment. **Supplementary worksheet 2**: Taxonomic annotation of the metagenomics sequences against the RefSeq database. **Supplementary worksheet 3**: Functional annotation of the metagenomics sequences against the SEED1 database. **Supplementary worksheet 4**: Output of the pair-wise statistical comparison between bulk soil and rhizosphere functional profiles. **Supplementary worksheet 5**: Output of the ANOVA among the functional profiles of the bulk soil and individual genotypes.

Additional file 4:

Supplementary worksheet 1: Information of completeness, contamination, heterogeneity, contig, size, coverage and taxonomic affiliation of the 28 high-quality MAGs.

884 ## Additional file 5:

885 **Supplementary worksheet 1**: Experimental design and dry weight data for the
886 conditioned soils experiment. **Supplementary worksheet 2**: Chemical and physical
887 parameters of the conditioned soil substrates. **Supplementary worksheet 3**: Output of the
888 environmental fitting analysis on chemical and physical properties of the plant-soil
889 feedback experiment.

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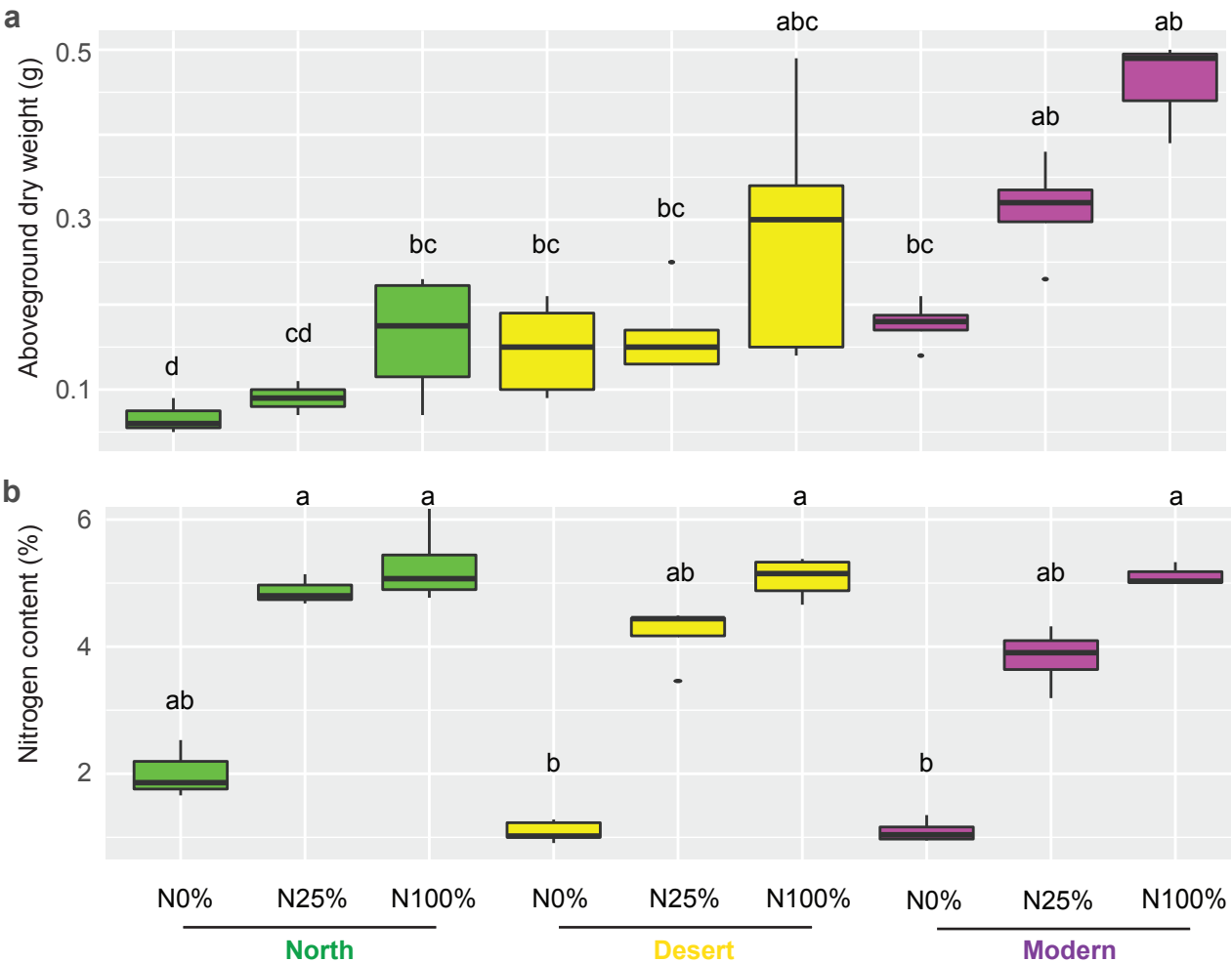
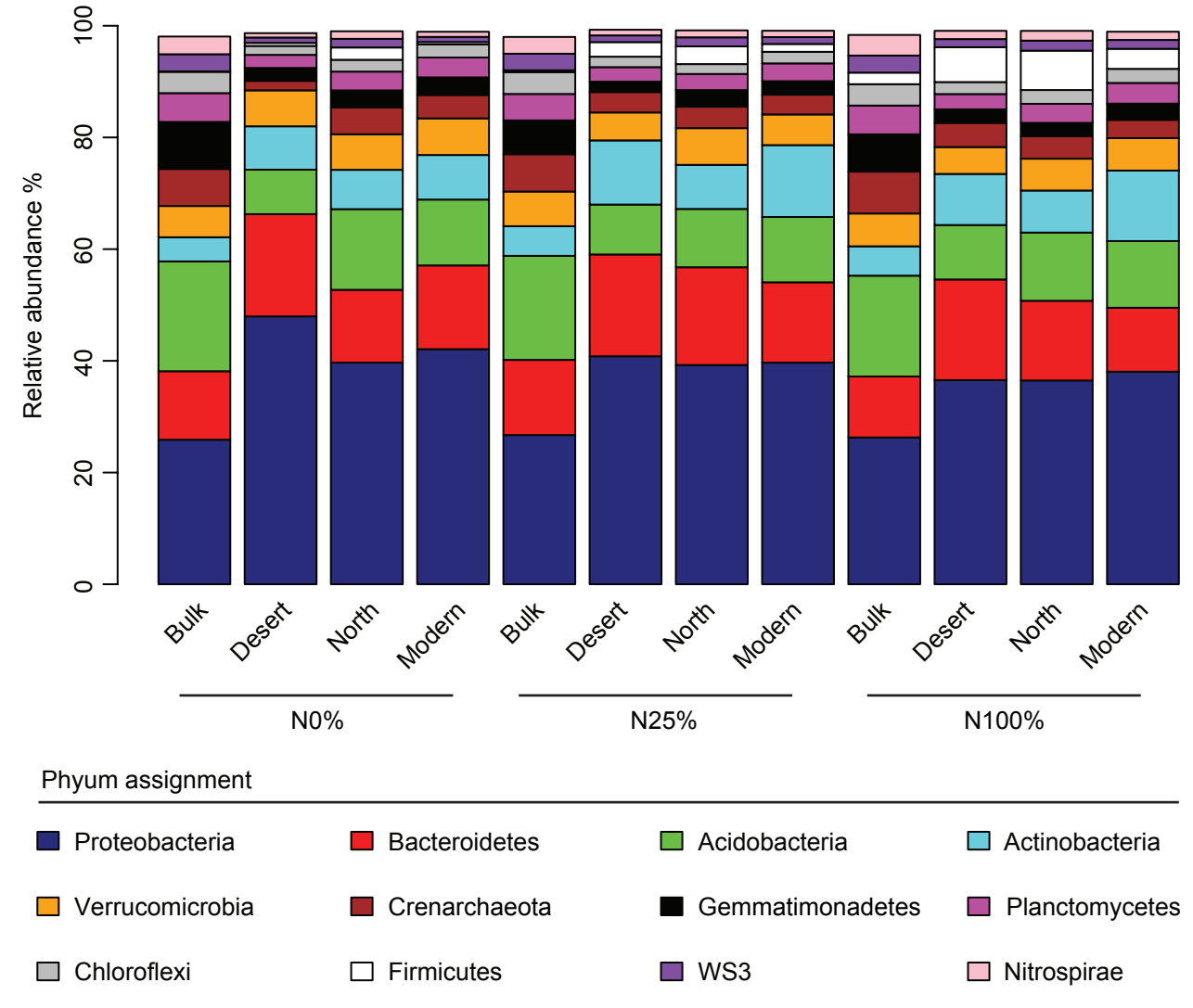
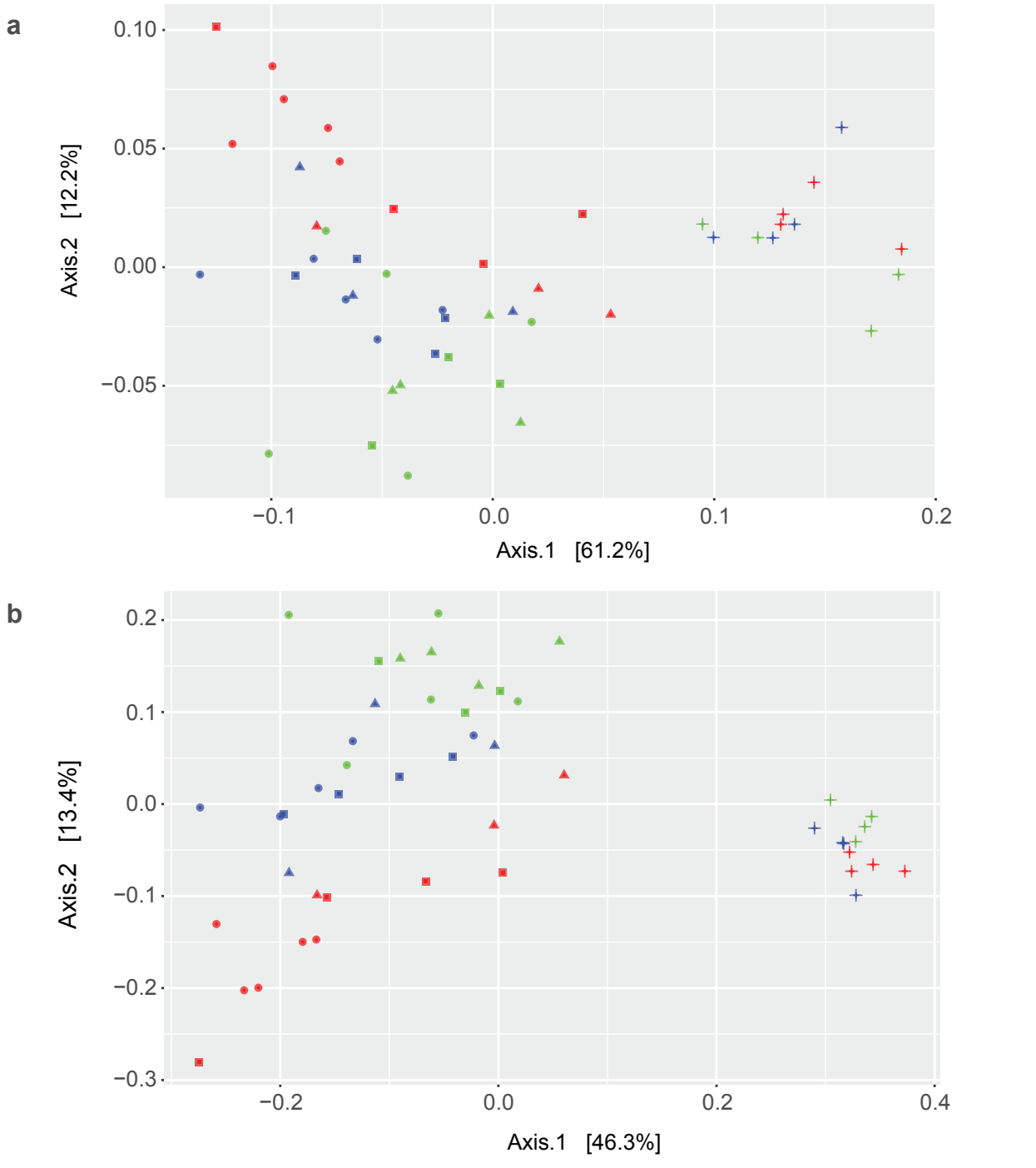
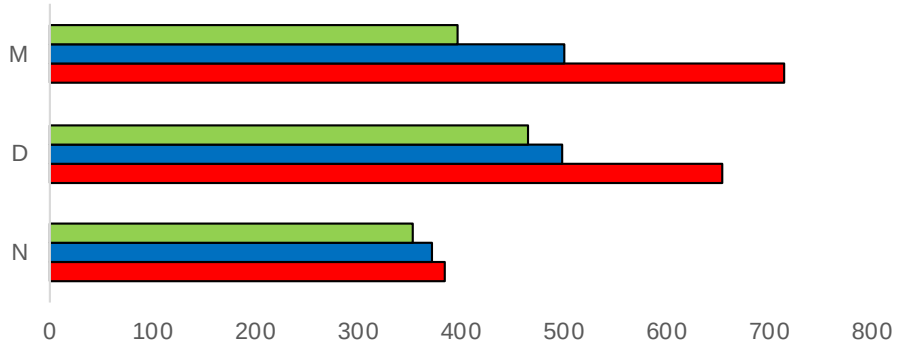

a
Aboveground dry weight (g)
0.5
0.3
0.1
d
cd
bc
bc
bc
abc
bc
ab
ab
b
Nitrogen content (%)
6
4
2
ab
a
a
b
ab
a
b
ab
a
N0%
N25%
N100%
N0%
N25%
N100%
N0%
N25%
N100%
North
Desert
Modern


Relative abundance %
0
20
40
60
80
100
Bulk
Desert
North
Modern
Bulk
Desert
North
Modern
Bulk
Desert
North
Modern
N0%
N25%
N100%
Phyum assignment
Proteobacteria
Bacteroidetes
Acidobacteria
Actinobacteria
Verrucomicrobia
Crenarchaeota
Gemmatimonadetes
Planctomycetes
Chloroflexi
Firmicutes
WS3
Nitrospirae

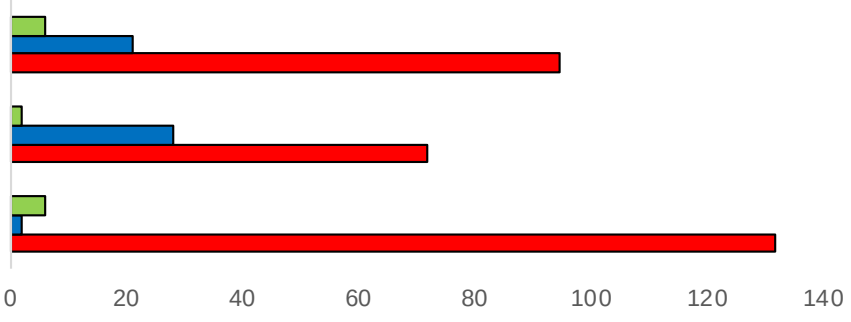

a
0.10
0.05
0.00
−0.05
Axis.2 [12.2%]
−0.1
0.0
0.1
0.2
Axis.1 [61.2%]
b
0.2
0.1
0.0
−0.1
−0.2
−0.3
Axis.2 [13.4%]
−0.2
0.0
0.2
0.4
Axis.1 [46.3%]
Treatment
N0%
N25%
N100%
Genotype
Bulk
Desert
North
Modern

a

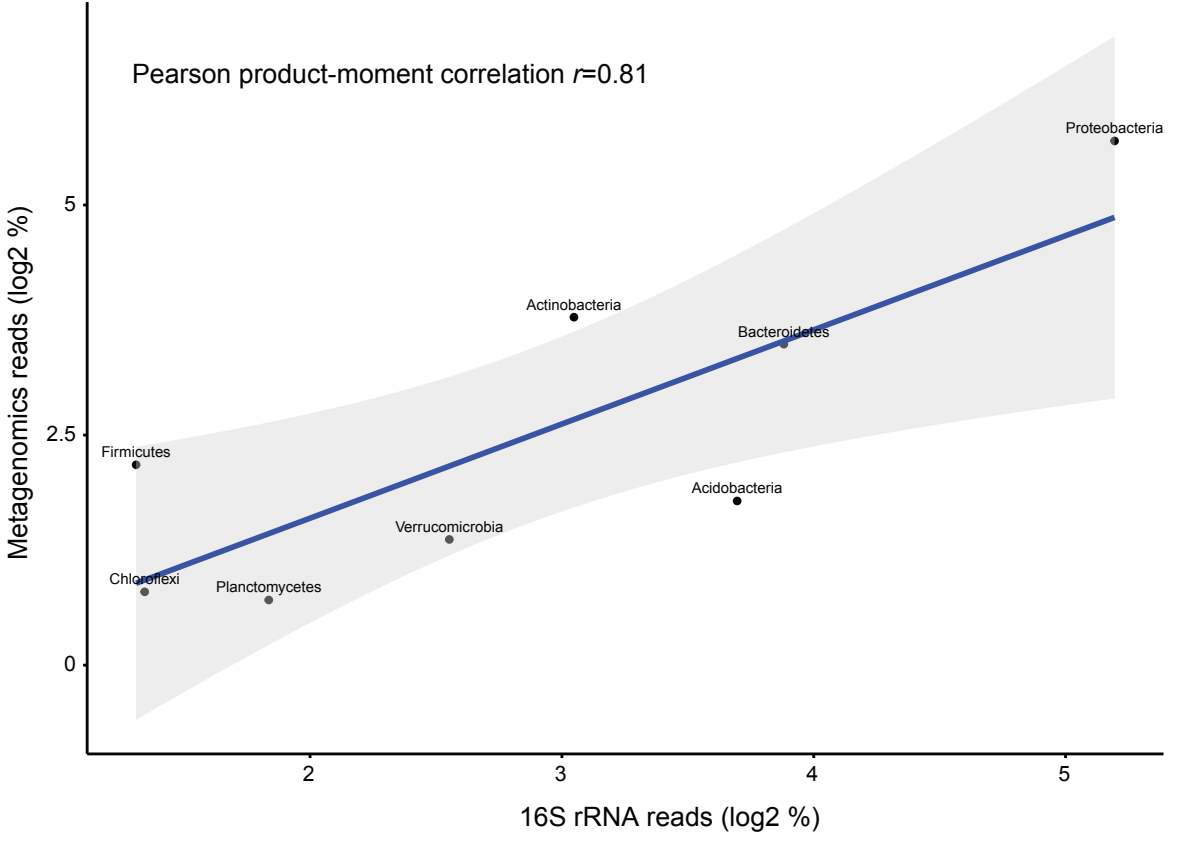


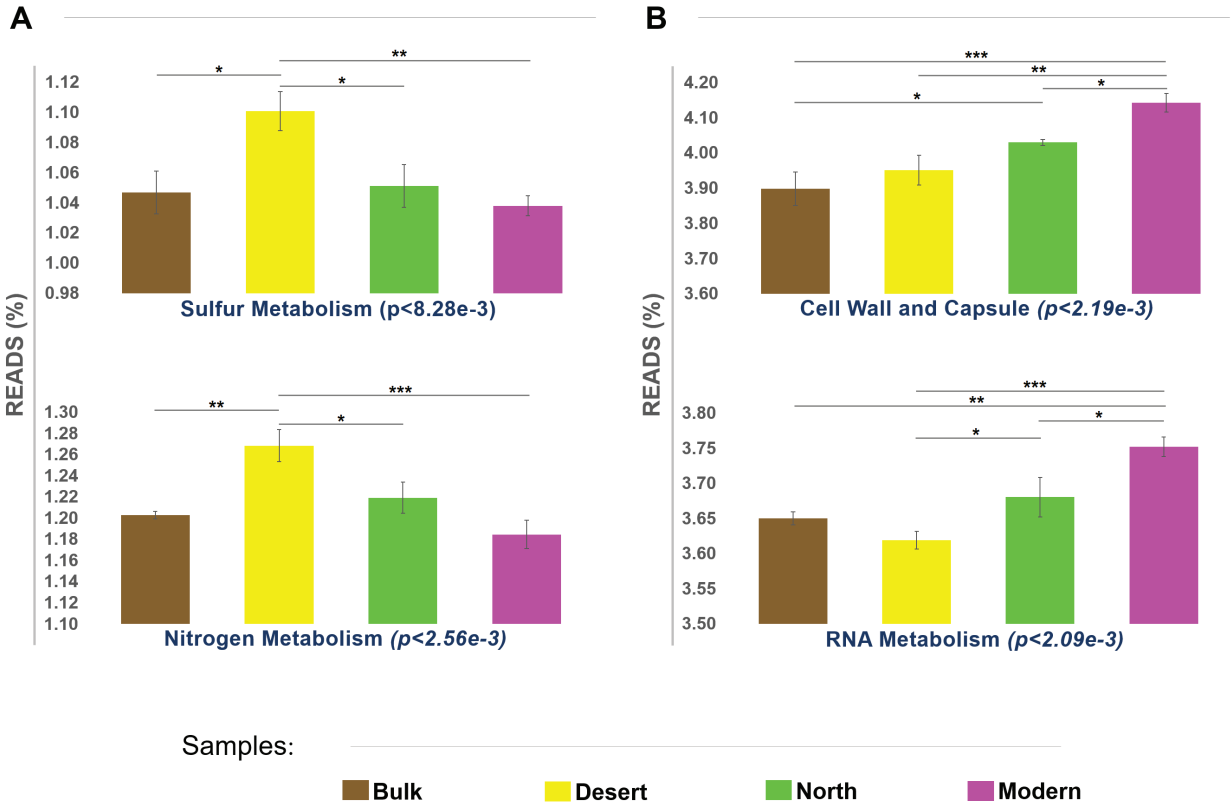
	N	D	M
100%	354	467	398
25%	372	499	501
0%	386	656	716

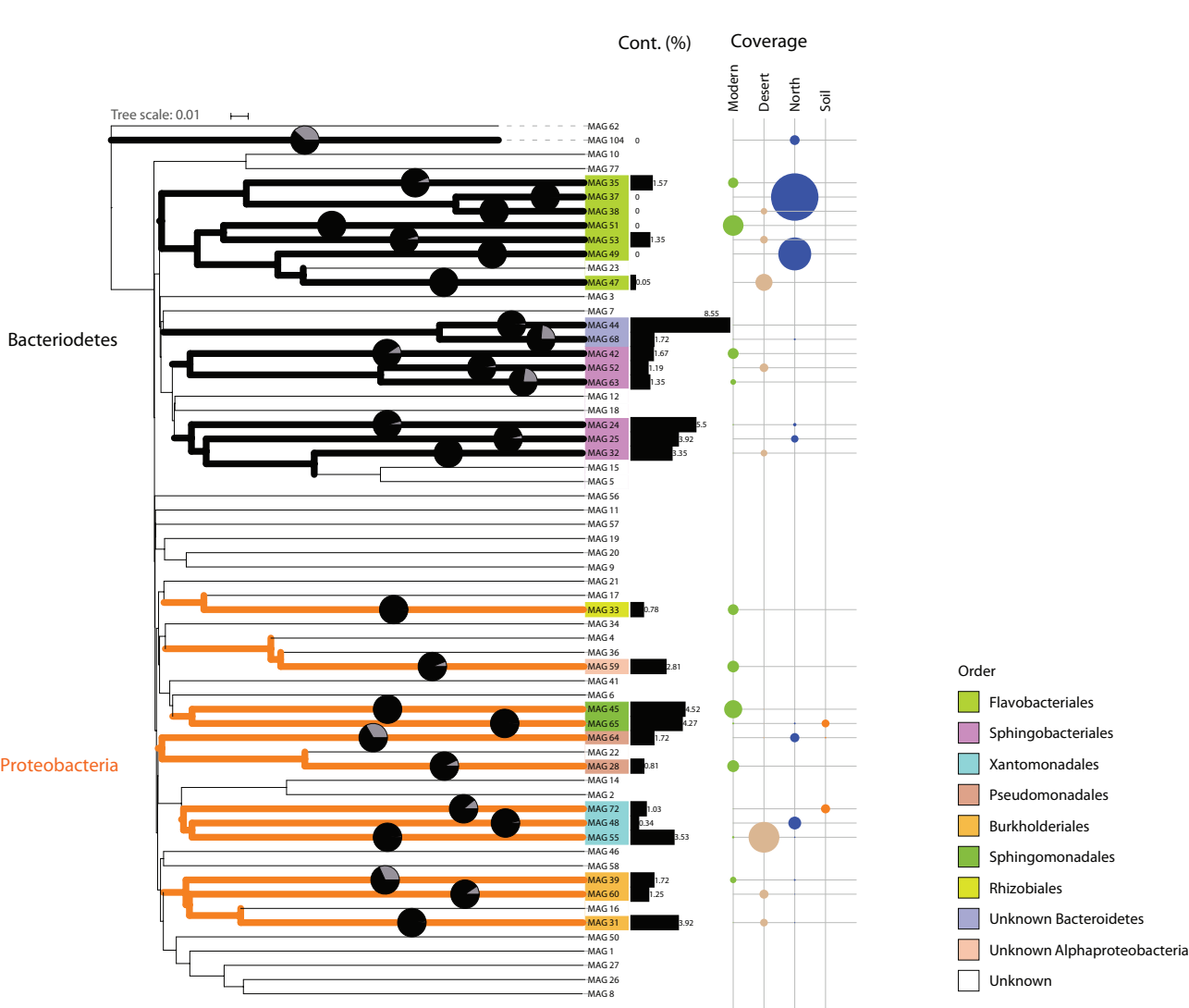
b

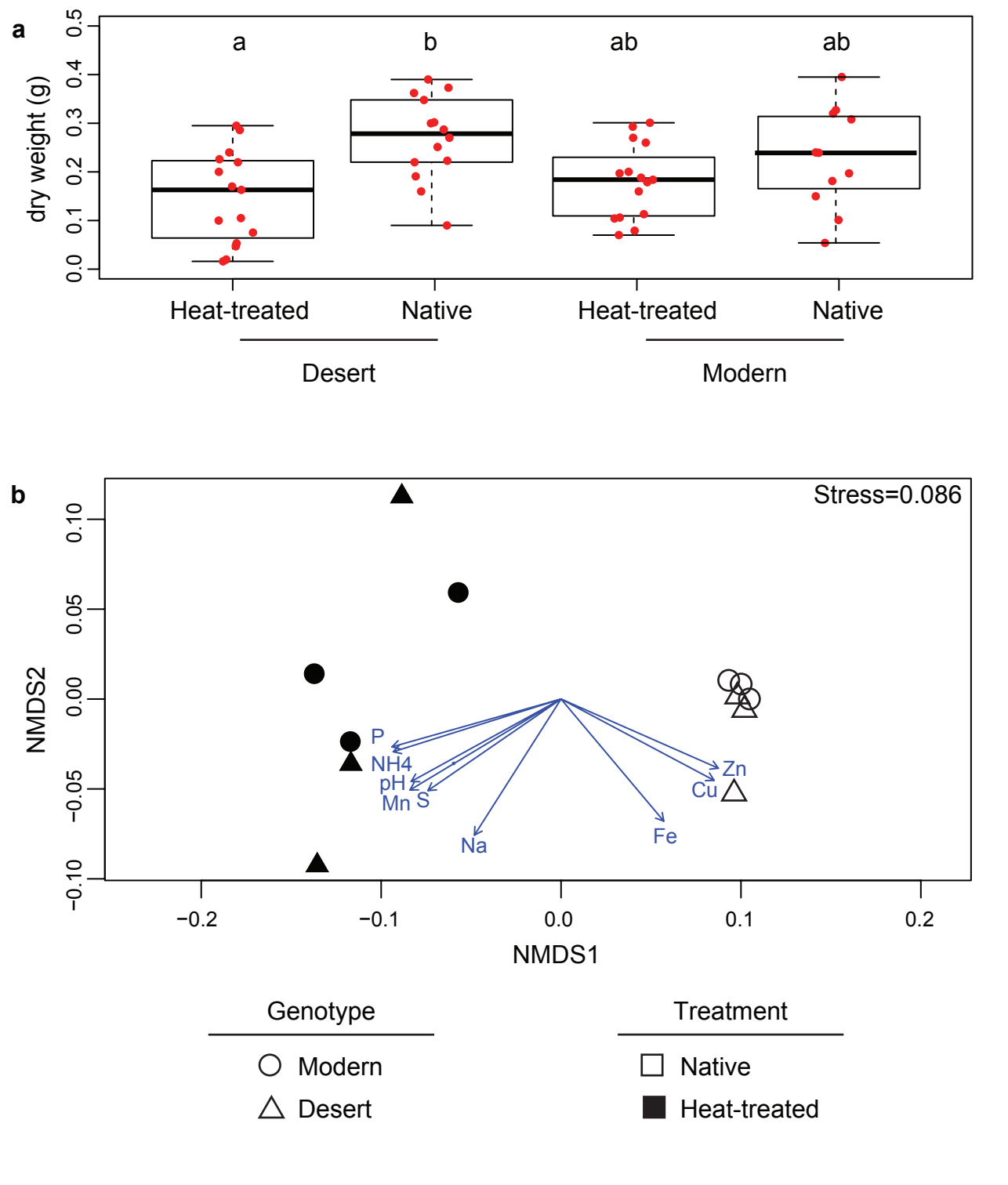


	N vs D	N vs M	D vs M
100%	6	2	6
25%	2	28	21
0%	132	72	95


Pearson product-moment correlation r=0.81
Proteobacteria
Actinobacteria
Bacteroidetes
Firmicutes
Acidobacteria
Verrucomicrobia
Chloroflexi
Planctomycetes
Metagenomics reads (log2 %)
16S rRNA reads (log2 %)
0
2.5
5
2
3
4
5


A
B
READS (%)
1.12
1.10
1.08
1.06
1.04
1.02
1.00
0.98
Sulfur Metabolism (p<8.28e-3)
1.30
1.28
1.26
1.24
1.22
1.20
1.18
1.16
1.14
1.12
1.10
Nitrogen Metabolism (p<2.56e-3)
4.20
4.10
4.00
3.90
3.80
3.70
3.60
Cell Wall and Capsule (p<2.19e-3)
3.80
3.75
3.70
3.65
3.60
3.55
3.50
RNA Metabolism (p<2.09e-3)
Samples:
Bulk
Desert
North
Modern


Cont. (%)
Coverage
Modern
Desert
North
Soil
Tree scale: 0.01
Bacteriodetes
Proteobacteria
MAG 62
MAG 104
MAG 10
MAG 77
MAG 35
MAG 37
MAG 38
MAG 51
MAG 53
MAG 49
MAG 23
MAG 47
MAG 3
MAG 7
MAG 44
MAG 68
MAG 42
MAG 52
MAG 63
MAG 12
MAG 18
MAG 24
MAG 25
MAG 32
MAG 15
MAG 5
MAG 56
MAG 11
MAG 57
MAG 19
MAG 20
MAG 9
MAG 21
MAG 17
MAG 33
MAG 34
MAG 4
MAG 36
MAG 59
MAG 41
MAG 6
MAG 45
MAG 65
MAG 64
MAG 22
MAG 28
MAG 14
MAG 2
MAG 72
MAG 48
MAG 55
MAG 46
MAG 58
MAG 39
MAG 60
MAG 16
MAG 31
MAG 50
MAG 1
MAG 27
MAG 26
MAG 8
0
1.57
0
0
0
1.35
0
2.05
8.55
1.72
1.67
1.19
1.35
5.5
3.92
3.35
2.78
2.81
4.52
4.27
1.72
2.81
1.03
0.34
3.53
1.72
1.25
3.92
Order
Flavobacteriales
Sphingobacteriales
Xantomonadales
Pseudomonadales
Burkholderiales
Sphingomonadales
Rhizobiales
Unknown Bacteroidetes
Unknown Alphaproteobacteria
Unknown


a
dry weight (g)
0.0
0.1
0.2
0.3
0.4
0.5
a
b
ab
ab
Heat-treated
Native
Heat-treated
Native
Desert
Modern
b
Stress=0.086
NMDS2
0.10
0.05
0.00
−0.05
−0.10
P
NH4
pH
Mn
S
Na
Fe
Zn
Cu
−0.2
−0.1
0.0
0.1
0.2
NMDS1
Genotype
Modern
Desert
Treatment
Native
Heat-treated