Interactions between soil compositions and the wheat root microbiome under drought stress: From an *in silico* to *in planta* perspective

Jiyeon Si [a,b,c,1], Emilie Froussart [d,e,1], Tom Viaene [f,1], Jorge F. Vázquez-Castellanos [a,b], Kelly Hamonts [f], Lin Tang [d,e], Stien Beirinckx [d,e], Annick De Keyser [d,e], Tibby Deckers [f], Fien Amery [g], Steven Vandenabeele [f], Jeroen Raes [a,b], Sofie Goormachtig [d,e,*]

[a] *Laboratory of Molecular Bacteriology. Department of Microbiology and Immunology, Rega Institute, Katholieke Universiteit Leuven, 3000 Leuven, Belgium*
[b] *Center for Microbiology, VIB, 3000 Leuven, Belgium*
[c] *Medical Science Research Institute, School of Medicine, Sungkyunkwan University (SKKU), Suwon 16419, Republic of Korea*
[d] *Department of Plant Biotechnology and Bioinformatics, Ghent University, 90e2 Ghent, Belgium*
[e] *Center for Plant Systems Biology, VIB, 9052 Gent, Belgium*
[f] *Aphea.bio NV, 90e2 Gent, Belgium*
[g] *Plant Sciences Unit, Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), 9820 Merelbeke, Belgium*

A R T I C L E   I N F O

A B S T R A C T

As wheat (*Triticum aestivum*) is an important staple food across the world, preservation of stable yields and increased productivity are major objectives in breeding programs. Drought is a global concern because its adverse impact is expected to be amplified in the future due to the current climate change. Here, we analyzed the effects of edaphic, environmental, and host factors on the wheat root microbiomes collected in soils from six regions in Belgium. Amplicon sequencing analysis of unplanted soil and wheat root endosphere samples indicated that the microbial community variations can be significantly explained by soil pH, microbial biomass, wheat genotype, and soil sodium and iron levels. Under drought stress, the biodiversity in the soil decreased significantly, but increased in the root endosphere community, where specific soil parameters seemingly determine the enrichment of bacterial groups. Indeed, we identified a cluster of drought-enriched bacteria that significantly correlated with soil compositions. Interestingly, integration of a functional analysis further revealed a strong correlation between the same cluster of bacteria and β-glucosidase and osmoprotectant proteins, two functions known to be involved in coping with drought stress. By means of this *in silico* analysis, we identified amplicon sequence variants (ASVs) that could potentially protect the plant from drought stress and validated them *in planta*. Yet, ASVs based on 16S rRNA sequencing data did not completely distinguish individual isolates because of their intrinsic short sequences. Our findings support the efforts to maintain stable crop yields under drought conditions through implementation of root microbiome analyses.

1. Introduction

As one of the most important staple foods cultivated worldwide, wheat (*Triticum aestivum* and *Triticum durum*) is of undeniable agronomic interest [1]. Currently, it is the second most-produced grain (29.11%) between maize (*Zea mays*) (42.51%) and milled rice (*Oryza sativa*) (18.88%) [2]. To meet the ever-increasing demands, enhancement of wheat yields has been a major objective of breeding programs for several decades [3–6]. Among the various abiotic and biotic stresses and within the current climate change, drought imposes one of the most serious constraints on plant harvests [7]. Since the 1970s, root-associated bacteria have been recognized for their potential to increase crop yields [8]. These beneficial microbes have been designated Plant Growth-Promoting Rhizobacteria (PGPR), and PGPR isolated from wheat rhizosphere have been reported to be effective in inorganic compounds solubilization, nitrogen fixation, and extracellular enzymes production [9,10].

Indeed, the root endosphere (i.e., the internal environment of the root) and the root rhizosphere (i.e., a narrow soil region next

* Corresponding author at: VIB-UGhent Center for Plant Systems Biology, 9052 Ghent, Belgium.
  E-mail address: sogoo@psb.ugent.be (S. Goormachtig).
  [1] Joint first author.

to and influenced by the root) harbor millions of microorganisms [11]. Recently, studies on the endosphere and rhizosphere microbiomes have revealed that drought stress modifies the community biodiversity and structure [12–14]. In particular, Actinobacteria are strongly enriched in the soil, rhizosphere, and endosphere environments of different plant species under drought conditions [15]. However, the importance of different soil parameters for the microbial community variations both under normal and stress conditions, such as drought, and the mechanistic links between various soil chemical properties, the microbial community, and the host plants are still underexplored [16–18]. The soil status is determined by various physicochemical and biological soil properties, such as organic carbon, total nitrogen, phosphate, pH, biomass, and water content [19]. Hence, understanding the associations of these properties with microbial communities can help enhance crop yields. For example, organic and inorganic amendments influenced the wheat microbial community by modifying soil properties and specifically, inorganic fertilizers increased the abundance of Actinobacteria and Bacteroidetes, whereas they decreased Proteobacteria [20]. The soil pH is also known as a main factor for the microbial community structure [21]. Yet, the relative importance of each of these drivers to the microbial communities requires additional research.

Whereas PGPR can alleviate drought stress, for example by upregulating drought-responsive genes,such as aquaporin (TaTIP1;1) and helicase genes [66] , plants have various other defense strategies to cope with it. For an efficient water use and further reduction of water loss, they reduce their leaf size and enlarge the root system more deeply into the soil [22]. They also depend on the production of a broad range of compounds for osmotic adjustment (e.g., proline, glycine betaine, and potassium), plant growth (e.g., abscisic acid, salicylic acid, auxins, and gibberellins), and antioxidation (e.g., polyamines and citrulline) [23]. Although these strategies are promoted by the plants, such a protection is also hinted at by bacterial contributions. For example, proline is one of the most important osmolytes found in the plant cytosol and is often used as a marker for drought stress. Under environmental stress, accumulation of proline is not only observed in higher plants, but also in algae, animals, and bacteria [24,25].

Here, we collected soils from six regions in Belgium and used them to plant three different wheat genotypes to better understand the simultaneous influence of edaphic (soil physicochemical and biological properties), environmental (drought treatment), and host (genotype) factors on the microbial communities in the unplanted soil and the root endosphere. We determined the association of soil parameters with the microbial community network, both in the soil and the endosphere. Finally, we could identify both *in silico* and *in planta* microbial strains that can potentially shield wheat plants against drought stress. Our findings provide an evaluation on how to translate changes in root microbial communities under adverse conditions and in different soil environments into potential PGPR for agriculture.

2. Materials and methods

2.1. Experimental design

Soil samples were collected from agricultural fields in six different regions in Belgium: Bassevelde (BA; sand), Bekkevoort (BE; sand/loam), Merelbeke (ME; sand), Poperinge (PO; sand/loam), Ravels (RA; sand), and Watervliet (WA; clay) as described previously [26] (Fig. 1A). Soil was sieved (0.5 × 0.5 cm, square-shaped holes) to remove large particles before use. For each soil type and wheat genotype, 10 conical PVC tubes (length 20 cm; diameter 5 cm) were used as technical replicates. In parallel, the tubes with-

out plants were kept for soil samples (87 g). Here, we defined the soil as unplanted soil. Soils were saturated with 30 mL of water per tube overnight before sowing. Commercially acquired seeds of three wheat genotypes, Intro (IN; winter variety), Tybalt (TY; spring variety), and Senatore Cappelli (CA; durum), were surface sterilized. For the surface sterilization, seeds were washed as follows: three times for 5 min with sterile water, 2 min with 70% (v/v) ethanol, 20 min with 9% (v/v) sodium hypochlorite solution (9 mL sterile water, 30 mL NaClO 12/13% [v/v] stock solution, and 1 mL Tween 20), and finally four times with sterile water for 15 min [26]. The seeds were dried in a Petri dish under a laminar flow and stored at 4 °C until utilization, whereafter they were pregerminated on germination paper for 48 h and transferred to conical tubes. In total, five seedlings were added per tube. The plants were watered with 30 mL of rainwater five times for 3 weeks, except for the drought-treated plants that received half the volume of the well-watered plants, because treatment induced drought-related wilting in the soils (Fig. S1). All plants were grown under the same conditions in the growth chamber (16 h of light:8 h of dark at 21 °C and 17 °C, respectively). After 3 weeks, the root (endosphere) and soil samples were collected, and the shoot biomass and soil properties were quantified. The soil pH (ISO 10390) and mineral nitrogen (NO_3^--N and NH_4^+-N; ISO 14256-2) were measured in 1 M potassium chloride (KCl) and total nitrogen (TN) and organic carbon (OC) by dry combustion (ISO 10694). Ammonium lactate extractable phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), sodium (Na), manganese (Mn), and iron (Fe) were assessed by extraction with ammonium lactate (S:L = 1:20, 4 h) [27]. Briefly, air-dried soil samples (5 g) were added to 100 mL of ammonium lactate. The extracts were shaken for 4 h in dark recipients before filtered and collected. The extractant ammonium lactate (1 L; pH = 3.75) was prepared by dissolving 88% lactic acid (25.7 mL), 99% acetic acid (23.4 mL), and 25% ammonium (16 mL) in distilled water. Additionally, the microbial biomass (bacteria and fungi) [28], soil moisture (%; weight of soil fractions before and after 3 days of drought at 60 °C × 100), and dry matter (DM) content (ISO 11465) were measured (Table S1). The microbial biomass was measured using phospholipid fatty acid (PLFA) analysis fv [67]. Root endosphere and soil samples were prepared as described previously [26]. Briefly, the soil samples were washed in phosphate-buffered saline (PBS) solution for 20 min and centrifuged at 3,220 × *g* for 20 min at 10 °C to ensure that all bacteria and soil particles were dissolved in the pellet. The supernatant was removed, and the remaining soil pellet was frozen in liquid nitrogen and stored at −80 °C. For the root endosphere sample collection, adhering soil was removed by shaking the roots vigorously. Roots were washed twice briefly in PBS, shaken in 500 mL sterile flasks with 50 mL PBS for 20 min, sonicated (10 min of 30-sec cycles at 4,000 Hz) to remove loosely attached organisms, flash-frozen in liquid nitrogen, and stored at −80 °C. Roots were ground in liquid nitrogen before DNA extraction. In total, 112 soil (two treatment conditions, six soil types, and eight to 10 replicates) and 336 endosphere samples (two treatment conditions, three genotypes, six soil types, and eight to 10 replicates) were prepared for further processing. Although our aim was 10 replicates, some were lost during the sample preparation.

2.2. 16S rRNA sequencing data preparation and processing

DNA was extracted from all collected samples with the DNeasy PowerSoil DNA kit (Qiagen, Hilden, Germany) and purified with AmPure Beads (Beckman Coulter, Pasadena, CA, USA). Next, the V4 region (515F-806R) of the 16S rRNA gene was amplified as proposed by the Earth Microbiome Project [29] and sequenced on HiSeq2500 (2 × 250 bp; European Molecular Biology Laboratory, Heidelberg, Germany). Sequencing data were first demultiplexed
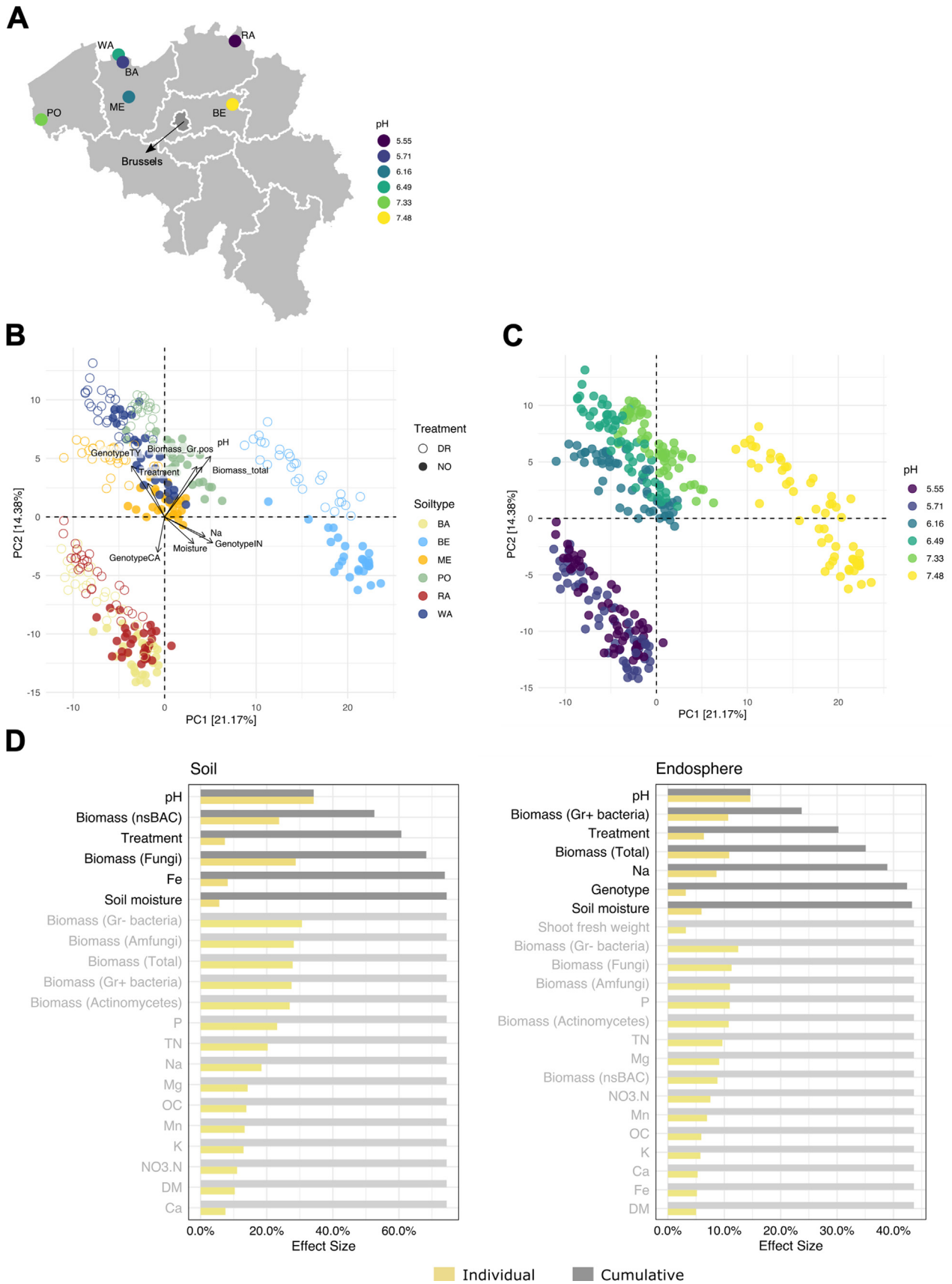
Fig. 1. Sampling regions and community variation in soil and endosphere. (A) Overview of sampling region in Belgium indicated with soil pH. BA, Bassevelde (sand); BE, Bekkevoort (sand/loam); ME, Merelbeke (sand); PO, Poperinge (sand/loam); RA, Ravels (sand from 'Kempen' location); WA, Watervliet (clay). (B-C) Principal component analysis of the root endosphere microbiome colored by drought treatment, soil type, and pH. Arrows indicate soil-related variables that can significantly explain the endosphere community variation. (D) Individual and cumulative effect size of soil-related covariates on microbiome community variations. Analysis was done without the BE soil. Bars indicate nonredundant cumulative and individual effect sizes. Variables labeled in grey indicate variables without additional contribution to the cumulative model. nsBAC, nonspecific bacteria; OC, organic carbon; TN, total nitrogen; DM, dry matter; Gr, Gram.

with LotuS 1.565 [30] and processed following the DADA2 pipeline [31]. Briefly, sequence reads were filtered and trimmed with the parameters: truncQ = 5, truncLen = c(230, 200), and trimLeft = c (10, 10). After denoising and removal of chimeras, an amplicon sequence variant (ASV; n = 53,496) table was constructed and taxonomy (n = 1,467) was assigned up to the species level with the GreenGenes version 13.8 [32]. Prior to the downstream analysis, we excluded taxa (i) unassigned to the kingdom Bacteria; (ii) assigned to the kingdom Archaea, class Chloroplast, and family Mitochondria; and (iii) low in abundance (read counts ≤ 5 in ≤ 5 samples). Subsequently, the taxonomy table was agglomerated to the genus level, yielding 833 and 864 taxa for the soil (n = 112) and endosphere samples (n = 336), respectively. When the taxon was not classified at the genus level, it was labeled to the nearest classified taxonomic levels available. The functional potential of the endosphere community was predicted with Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2-2.3.0_b) following to default parameters [33]. Prior to the downstream analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs (KOs) included in KEGG pathways of human diseases were excluded. Bacterial and functional potential abundances were transformed by means of the centered log-ratio (CLR) transformation to control the compositionality of the sequencing data (*CoDaSeq* R package function *codaSeq.clr*) [34]. Zero imputation prior to the log transformation was implemented by means of the minimum proportional abundance for each taxon [35].

2.3. Microbial modules in soil and endosphere communities

Bacterial interactions and associations with soil properties were analyzed by means of the weighted correlation network analysis (WGCNA) on the CLR-transformed abundance, including every taxon classified up to the genus level [36]. A signed adjacency matrix was calculated with the soft-thresholding power ($\beta = 7$), fitting the scale-free topology network. Subsequently, the matrix was converted to a Topological Overlap Matrix to define bacterial modules (minModulesize = 50 and 30 for the soil and the endosphere, respectively) with hierarchical clustering (average linkage). Association of the bacterial modules with soil properties was determined by correlating the eigenvector of each cluster and the variables. The network was plotted with Gephi 0.9.2 [37]. Adjacency threshold was set to 0.08 to filter edges.

2.4. Collection of bacterial wheat isolates

The isolation of the *Streptomyces* strain was attempted as follows: 10 plants of the three wheat genotypes were grown under drought conditions for 3 weeks in conical PVC tubes as indicated below. Root samples were sterilized and crushed as described [26]. Subsequently, the diluted suspensions were plated on four different bacterial media (ATCC-2, TSB, ISP4, and ISP7) that are commonly used to isolate Actinobacteria (for the media composition, see Supporting Protocol) and incubated at 21 °C. A selection with nalidixic acid (20 μg/mL) allowed the enrichment of Gram-positive bacteria. Single colonies were picked based on colony morphology (for instance, resembling *Streptomyces* and streaked until pure cultures). Each strain was subjected to full-length 16S rDNA Sanger sequencing (Supporting Protocol) followed by NCBI BLAST search for the taxonomy information. The obtained 16S rRNA sequences of all strains used are presented in Table S2.

2.5. Screening of growth-promoting root isolates

Seeds of the IN variety were surface sterilized and pregerminated for 48 h on PBS solution. The growth promotion of each

strain was tested in two or three repeats. The overnight *Streptomyces* culture (30 mL) was briefly centrifuged and resuspended in 20 mL of PSB buffer prior to inoculation. For each repeat, seven seedlings were inoculated by shaking on an orbital shaker (Belico, Vineland, N.J., USA) and in a bacterial solution for 3 h. As a negative control, seedlings were inoculated with PBS buffer (mock treatment) for 3 h. Plants were cultivated in separate conical PVC tubes; all tubes per treatment were put together in the same tray. Inoculated or mock-treated seedlings were sown in potting soil (1:5 v/v mixture; Saniflor® potting soil with high NPK 8 kg/m^3 and low NPK 2 kg/m^3; NV Van Israel, Geraardsbergen, Belgium).

2.6. Statistical analyses

Combined explanatory power (effective size) of soil-related variables pooled in categories were calculated with the bioenv function in the *vegan* R package (version 2.5–5) [38]. Explanatory power of the individual variables on the microbial community variation was determined with distance-based redundancy analysis in *vegan*. Subsequently, nonredundant cumulative explanatory power of the variables was calculated by a forward stepwise model using the ordiR2step function in *vegan*. This analysis identifies nonredundant covariates that can explain the community variation through model fitting and provides their cumulative effect size. These nonredundant covariates were added as arrows with the envfit function in the *vegan* R package. Community diversities (α- and β-diversity) were analyzed with *phyloseq* (version 1.28.0) and *vegan* R packages [38,39]. Prior to the analysis, samples were rarefied to 85,000 and 10,000 reads for the soil and endosphere microbiome, respectively. When the soil and endosphere community diversities were compared, the soil samples were rarefied to 10,000 reads for direct comparison. All other analyses were done with the CLR-transformed abundance table. The top 10 taxa contributing to the community variation were identified with the envfit function and presented as arrows. Group means were compared with the Wilcoxon rank sum test. When more than two groups were compared, the Kruskal-Wallis test was applied, followed by the post-hoc Dunn test. Multiple testing correction was applied by the Benjamini-Hochberg method (reported as FDR).

3. Results

3.1. Covariates associated with the soil and endosphere microbiomes

To characterize the microbial community in the soil and endosphere, we first analyzed the associations of the different physico-chemical and biological soil properties, soil type, genotype, and conditions (i.e. well-watered vs. drought-treated) with the microbial community variations in the endosphere (Fig. 1). Here, we defined soil type as the region where the soil had been isolated. Principal component analysis based on CLRtransformation at every taxonomic level classified up to the family level revealed that the endosphere community was affected by the drought treatment (Adonis R^2 = 0.06, P < 0.001) and soil type (Adonis R^2 = 0.42, P < 0.001; Fig. 1B). Also, a weak, but significantly, different microbial assemblage occurred among the wheat genotypes after deconfounding soil types and treatment (Adonis R^2 = 0.03, P < 0.001; Fig. S2A). Similarly, as for the endosphere, the soil community was also distinguished by drought treatment (Adonis R^2 = 0.06, P < 0.001) and soil type (Adonis R^2 = 0.74, P < 0.001; Fig. S2B).

Secondly, we took the analyzed soil characteristics into account to understand the differences between the endosphere and soil microbiomes and the extent of their effect sizes for the elucidation of the microbial community variations. To this end, we first calculated the explanatory power of the covariates pooled in categories

on the microbiome composition variations (Fig. S2D and Table S3). The variations in both the soil and endosphere microbiomes largely depended on edaphic (soil physicochemical and biological properties) factors. Next, we implemented a distance-based redundancy analysis (dbRDA) followed by a forward stepwise model selection, which determined the individual covariate and nonredundant cumulative effect sizes. Here, the variables corresponding most with the community variations in the root endosphere and soil communities were the soil regions and types (adjusted R^2 range = 37.4%–72.6% for soil and adjusted R^2 range = 19.85%–41.0% for endosphere; false discovery rate [FDR] < 0.1). Given that these variables masked the explanatory power of the physicochemical and biological soil properties, we subsequently analyzed them by excluding the soil regions and types and found that the soil pH was the highest explanatory power for both communities (adjusted R^2 range = 14.6%–34.2%, FDR < 0.1; Fig. 1D; Table S4). The soil pH impact on the microbial communities was also confirmed by the ordination analysis (Adonis R^2 = 0.16, $P < 0.001$ for the endosphere and Adonis R^2 = 0.34, $P < 0.001$ for the soil; Fig. 1C and Fig. S2C). Prior to this multivariate analysis, the BE soil was further excluded because of its high calcium levels and pH values with a confounding effect (maximum 9-fold difference between minimal and maximal Ca values) as a consequence. In addition to the pH, microbial biomass, drought treatment, sodium (endosphere only), iron (soil only), wheat genotype (endosphere only), and soil moisture were significant nonredundant variables for the microbiota community variations (adjusted R^2 range = 5.0%–23.7%, FDR < 0.1; Table S4). Whereas 3.2% of the community variation in the endosphere depended on the wheat genotype, the drought treatment similarly affected the soil and the endosphere community (7.4% and 6.4%, respectively; FDR < 0.1). Covariate analysis of the significant parameters and the community composition revealed a positive trend for soil pH and biomass (i.e., total and Gram-positive bacterial biomass for the endosphere community and fungal biomass for the soil community) (Fig. 1B and Fig. S2B).

3.2. Comparison of soil and endosphere microbiomes under well-watered conditions

The microbial community structure significantly varied between the endosphere and the soil. After exclusion of the drought-treated samples, the microbial composition between the two communities differed significantly (Adonis R^2 = 0.32, $P < 0.01$; Fig. 2A). The community richness was significantly higher in the soil than that in the endosphere across all different soil types (Mann–Whitney U [MWU] test, FDR < 0.01; Fig. 2B). All the phyla found in the endosphere community were also present in the soil, but the latter had 10 extra phyla. Out of 32 phyla, 26 differed significantly in their abundance between the two compartments (Fig. 2C; Table S5). For example, Proteobacteria, Bacteroidetes, and Actinobacteria were among the dominant phyla in both compartments, but the relative abundance was significantly greater in the endosphere (MWU test, FDR < 0.1) than in the soil. Examination of the taxa correlated with the community composition revealed that, based on the soil pH, the soil and endosphere communities had different contributors (Fig. 2D; Table S6). Dominant phyla, Bacteroidetes and Verrucomicrobia, increased in the soil community with moderate pH levels (6.16–7.33). Similarly, Actinobacteria, Acidobacteria, and Chloroflexi were associated with moderate pH levels in the endosphere community. In both soil and endosphere communities, low-abundant phyla, AD3 and FCPU426, increased in acidic soils with pH levels of 5.55–5.71. The effect of different wheat genotypes on the community composition was relatively modest, because 50% of the whole community

did not significantly differ between the various genotypes (Kruskal-Wallis test, FDR greater than 0.1; Table S7).

3.3. Impact of drought treatments on the wheat endosphere microbiome

Drought treatments hindered wheat growth in most soil types (with the exception of the BE and ME soils; Wilcoxon rank sum test FDR < 0.1; Fig. S3A). Wheat shoots harvested in WA soil had the highest fresh weight, both under drought and well-watered conditions. Especially, the shoot fresh weight of IN and TY wheat genotypes was higher when grown in WA soil than in other soils (Kruskal-Wallis test, FDR < 0.1; Fig. S3B). The percentage of water reduction under drought conditions varied among the soil types (Fig. S3C; Table S8). As soil moisture of the PO and WA soils was already lower than that of other soils, even before the drought treatment, the influence of the drought treatment was not as high, given that the loss in water content was<50% compared to approximately 70% or more in the other soils.

The response of the community diversity to the drought treatment was opposite for the soil and the endosphere (Fig. 3A). In the soil, the community richness at the family level significantly decreased across all soil types (MWU test, FDR < 0.01), whereas it increased in the root endosphere community (MWU test, FDR < 0.05). Yet, the increment in community richness in the root endosphere depended on the soil, because the increases were not significant in the PO and WA soils that had lost less water during the drought treatment, probably the reason for the less drastic effects on the root endosphere richness. Furthermore, the community richness augmented significantly in the roots of the CA and IN, but not TY, wheat genotypes (Fig. S4B). The Shannon index (community biodiversity) of the soil mostly corresponded with the overall community richness in the soil community, except for the BE soil, in which the biodiversity was enhanced under drought (Fig. S4A). In the root endosphere community, only the BA and BE soils varied significantly under the conditions. Based on the wheat genotype, the Shannon index was significantly reduced in the IN genotype, which could be attributed to a decrease in evenness by the increase in rare taxa (Fig. S4B). In a previous analysis, the IN genotype pointed in the same direction as the soil moisture, hinting at the association of the genotype with an adequately water-provided environment (Fig. 1B), in turn, making a fostering environment available for the broad range of bacteria, including the rare taxa.

Subsequently, we analyzed the effect of drought treatment at the phylum level in the soil and the endosphere microbiomes. The degree of microbial shifts was comparable between the soil and endosphere microbiomes. Out of 30 phyla, 23 were significantly different under the drought treatment (Table S9A). In the root endosphere community, 19 out of 28 phyla differed significantly between the two conditions with five intersecting phyla across all soil types (i.e., Actinobacteria, unclassified Bacteria, Fibrobacteres, WS3, and BRC1; Fig. S5A; Table S9B). Among the highly abundant phyla, Actinobacteria were enriched across all soil types, but Fibrobacteres were depleted by drought. Unlike the endosphere community, Proteobacteria were enriched under drought conditions across all soil type samples (Fig. S5B). The soil and endosphere communities shared seven and five phyla enriched and depleted under drought conditions, respectively (Fig. 3B). Among the highly abundant and shared phyla between the two communities, Actinobacteria, Chloroflexi, Acidobacteria, and Gemmatimonadetes were drought enriched, but Fibrobacteres generally drought depleted.

Analysis at the ASV levels identified 1,722 significant drought-responsive ASVs in the root endosphere community (Table S10; Wilcoxon rank sum test, $P < 0.05$ and FDR < 0.1). Most of the signif-
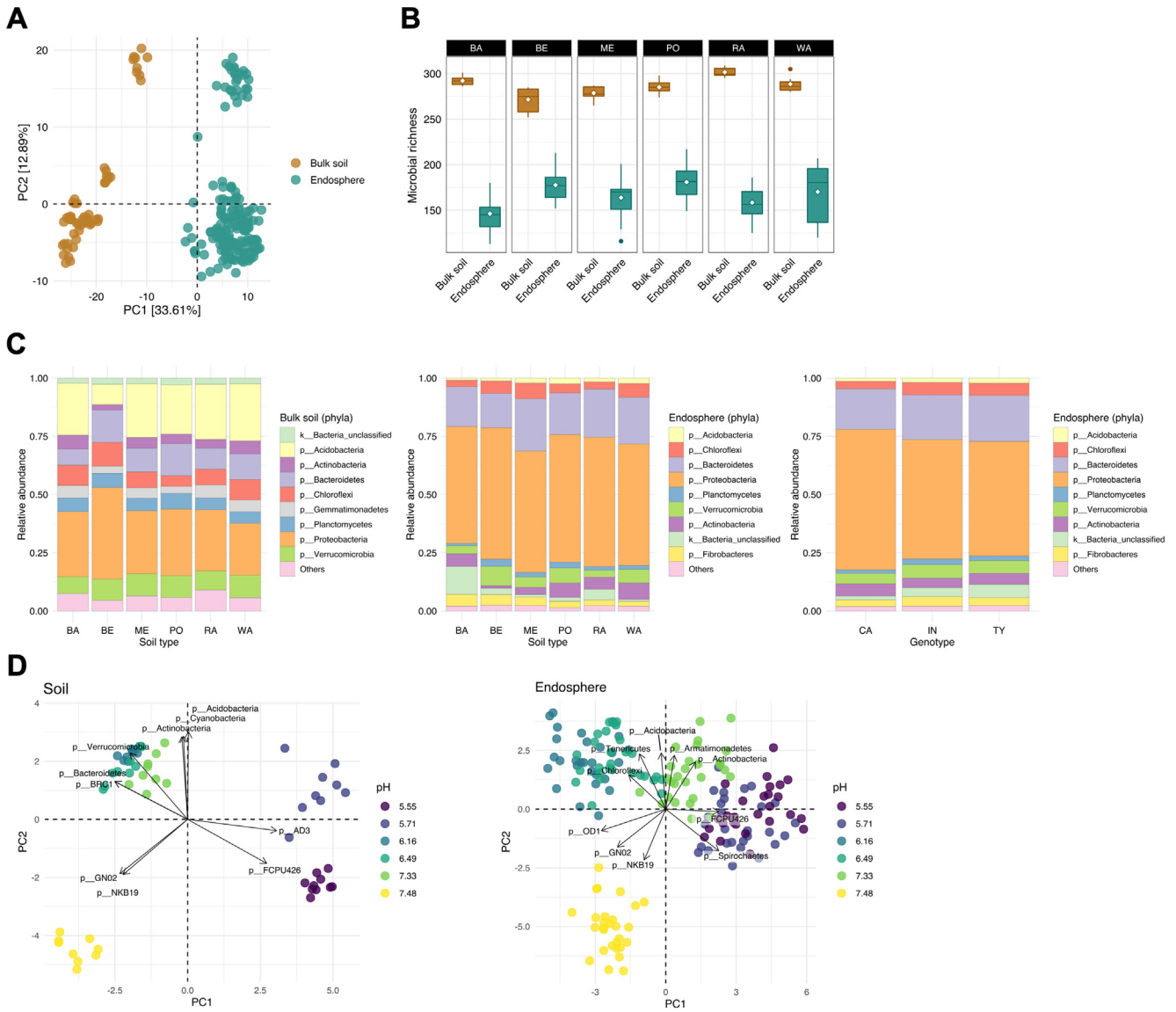
Fig. 2. Comparison of soil and endosphere microbiomes under well-watered condition. (A) Principal component analysis of the two compartments. (B) Community richness across different soil types. (C) Bacterial relative abundances at the phylum level. Left: soil community by soil type. Middle: endosphere community by soil type. Right: endosphere community by genotype. (D) Principal component analysis with top 10 phyla (arrows) significantly contributing to the community variations. BA, Bassevelde (sand); BE, Bekkevoort (sand/loam); ME, Merelbeke (sand); PO, Poperinge (sand/loam); RA, Ravels (sand from 'Kempen" location); WA, Watervliet (clay).

icant ASVs were drought depleted (73.9%) and 26.1% were enriched upon drought. Of the major taxa (top 10 most abundant phyla, families, and genera), microbial shifts of Proteobacteria (total ASV n = 669), Bacteroidetes (total ASV n = 408), Verrucomicrobia (total ASV n = 132), and Planctomycetes (total ASV n = 38) were not detected under drought at the phylum level (total ASV n = 1,676), but the dominant proportion of their ASVs were drought depleted (Fig. 3C). The drought response indicated the highest consensus for the ASVs matching Actinobacteria (78.1% of drought-enriched ASVs; total ASV n = 151) and Fibrobacteres (96.2% of drought-depleted ASVs; total ASV n = 26). Chloroflexi (total ASV n = 95), Acidobacteria (total ASV n = 44), and Gemmatimonadetes (total ASV n = 7) were drought enriched at the phylum level, but more than 70% of their ASVs were drought depleted. At the lower taxonomic level, the rate of consensus was higher. For example, the major ASV numbers of Comamonadaceae (total ASV n = 68), Hyphomicrobiaceae (total ASV n = 32), Polyangiaceae (total ASV n = 26), and A4b (total ASV n = 53), which were significantly depleted under drought stress, were also drought depleted (73.5

– 92.3%). At the genus level (total ASV n = 791), the ASVs of *Agrobacterium* (total ASV n = 10), *Cellvibrio* (total ASV n = 16), *Methylibium* (total ASV n = 8), *Devosia* (total ASV n = 9), *Opitutus* (total ASV n = 38), and *Fluviicola* (total ASV n = 18) corresponded (70.0 – 100.0%). Most ASVs matching Actinobacteria were consistently drought enriched at their lower taxonomic levels (e.g., family Streptomycetaceae and genus *Streptomyces;* 71.4 % and 70% of ASVs, respectively; Fig. S5C).

3.4. Microbial modules in the endosphere community

To determine microbe-microbe interactions and their associations with different soil properties in the endosphere, we analyzed the bacterial co-occurrence by WGCNA and identified four bacterial modules that we defined with a color code, corresponding with the physicochemical and biological soil property gradients of the soils in which the roots were grown (Fig. 4A and Table S11). The Green module contained the largest number of taxa (n = 146) followed by the Blue (n = 132), Orange (n = 97), and Red (n = 76).
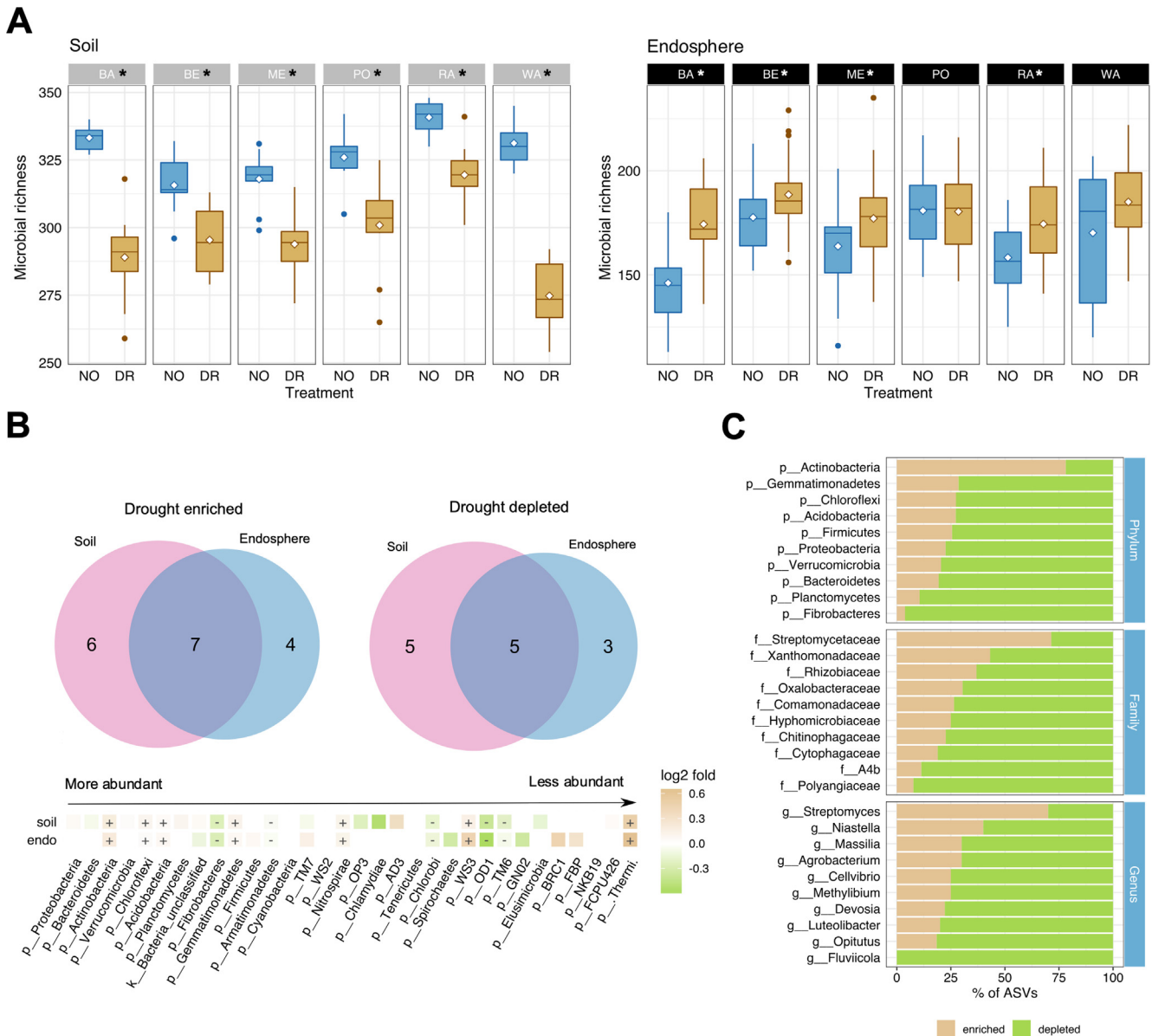
Fig. 3. Drought treatment response in soil and endosphere microbiomes. (A) Community diversity at the family level across all soil types. *, FDR < 0.1. (B) Overlap of drought-responsive phyla between the soil and the endosphere community. + and – indicate drought-enriched and drought-depleted taxa examined in both compartments. (C) Drought-responsive ASVs in the endosphere community. The 10 most abundant strains at the phylum, family, and genus level are shown.

Although the Red and Orange modules had an overall discordant relationship with their soil properties, they share negative trends with soil moisture, organic carbon, calcium, and sodium and positive with nitrogen (nitrate-nitrogen and total nitrogen) and dry matter (Pearson correlation, FDR < 0.1). Moreover, the two modules consisted of an enhanced proportion of drought-enriched taxa, defined to be significantly higher in their abundance under the drought treatment than the Blue and Green modules (Fig. 4B; Tables S11-S15). In the Orange module, 83.5% of the taxa were drought enriched, whereas the Red, Green, and Blue modules contained 75.0%, 21.9%, and 19.7% of drought-enriched taxa, respectively. As the Actinobacteria had been found to be consistently drought enriched, their nodes were colored blue to indicate whether or not, they overlapped with the two modules composed of drought-enriched taxa (Fig. 4A). A distinct clustering occurred where Actinobacteria were concentrated at the locations of the

Red and Orange modules, whereas non-Actinobacteria were mostly found at the Blue and Green module locations. Application of the same method for the soil community revealed similar trends with the Orange (100%) and Red (62.1%) modules that contained the largest number of drought-enriched taxa (Table S16). These two modules also shared the same course for nitrate-nitrogen (+), calcium (-), and sodium (-) as the endosphere modules, implying that these soil compositions are important for drought-resistant bacteria (Fig. S6).

3.5. Drought-enriched functional potential in the endosphere community

To investigate potential functional changes associated with drought stress and their associations with the bacterial modules, we first identified 205 significant drought-responsive KOs in the
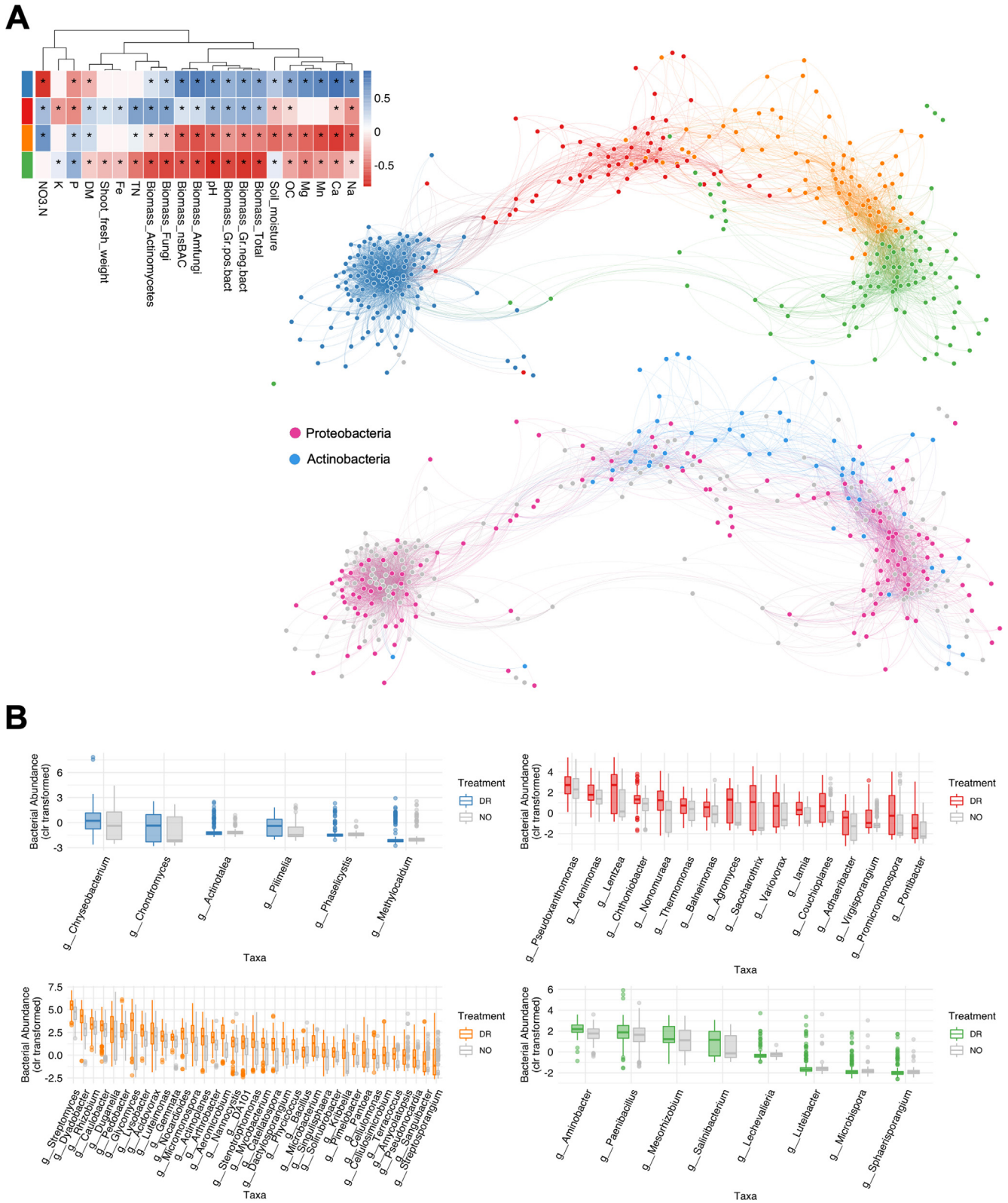
Fig. 4. Co-occurrence analysis of the endosphere community. (A) Nodes colored for each module (top; green, blue, orange, and red) and phylum (bottom; pink, Proteobacteria; blue, Actinobacteria; and grey, others). Correlations between bacterial modules and physicochemical and biological properties of soil are presented as a heatmap. OC, organic carbon; TN, total nitrogen; DM, dry matter. *: FDR < 0.1. (B) Drought-resistant genera in different modules. All are significant. NO, well-watered; DR, drought treated. Module color matches in all Figures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

endosphere community (Wilcoxon rank sum test FDR < 0.05; Table S17). Out of 205 KOs, we focused on KOs enriched under drought stress and analyzed their associations with the four bacterial modules (Spearman correlation, FDR < 0.1; Fig. 5). As expected, the Orange (46.63%) and Red (41.17%) modules presented a higher rate of drought-enriched KOs than that of the other two modules
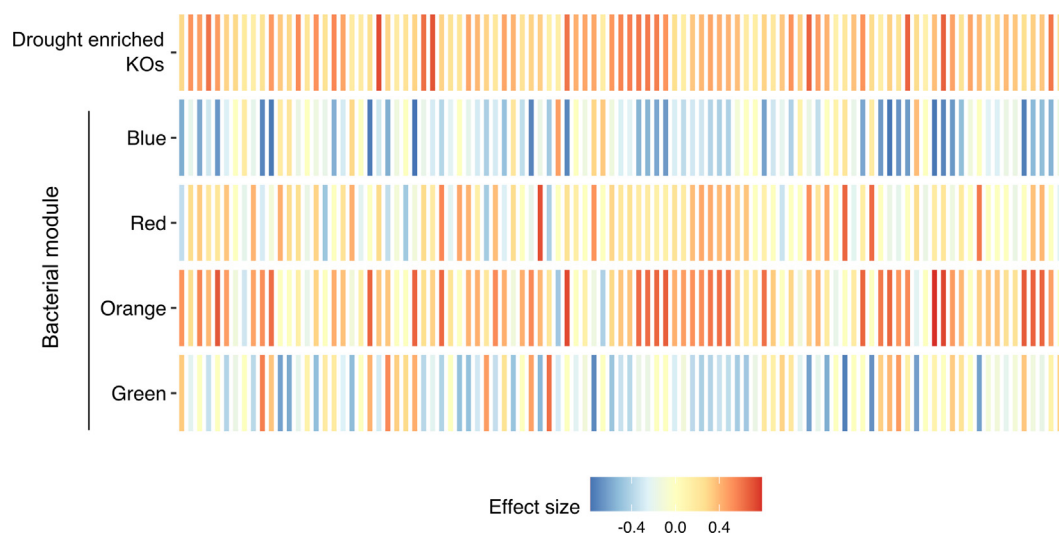
Fig. 5. Predicted functional potential for the endosphere community. Drought-enriched KOs were determined by comparing the enzyme abundance between the conditions (drought treatment). Correlations between the enzymes significantly enriched under drought stress and the bacterial modules were subsequently analyzed (FDR < 0.1).

(25.66%, and 12.75% for the Green and Blue, respectively). These results functionally support the drought-responsive bacterial modules and potential drought-resistant mechanisms induced by the endosphere bacteria.

3.6. Growth-promoting bacterial isolates of wheat under drought stress

Based on the *in silico* analysis, we hypothesized that bacterial genera, of which the relative abundance is enhanced during drought treatments, could trigger adaptation to drought stress and promote wheat growth under this condition [16–18]. To this end, we focused on *Streptomyces* that presented the highest number of matching ASVs enriched under drought stress and was the most abundant bacteriium in the Orange module. A total number of 14 *Streptomyces* strains were isolated from the root endosphere and screened for growth-promoting effects on juvenile wheat under drought conditions (Fig. S7).

We measured plant height, shoot dry and fresh weights, and the wilting status (i.e., upright or flat) as evaluation criteria for growth promotion (Fig. 6A). A small significant increase in plant height (106–111% compared to mock-treated plants; Wilcoxon rank sum test, $P < 0.05$) was observed when the plants were inoculated with three *Streptomyces* strains (DRGH_6, DRGH_13, and DRGH_14; Table S18). Furthermore, the shoot fresh weight significantly increased after inoculation with DRGH_14 under drought stress (140% compared to mock-treated plants; Wilcoxon rank sum test, $P < 0.05$). However, the shoot dry weight did not change by any bacterial treatment under drought conditions. Additionally, whereas the noninoculated plants wilted because of vigor loss upon the drought treatment, inoculation with one *Streptomyces* strains (DRGH_7; *Streptomyces* sp.) prevented the juvenile wheat plants to wilt (Fig. 6B).

To determine whether these protective isolates were significant at the sequence level, we compared their Sanger-inferred sequences with ASVs determined from the endosphere community from our dataset (Table S19). Within the group of strains that protected wheat plants upon drought treatment, three strains (DRGH_6, DRGH_13, and DRGH_14) were fully covered by ASVs (ASV172 and ASV715) that were significantly enriched upon drought treatment. However, ASV715 was also represented by isolates without any protective effect upon drought treatment

(DRGH_5 and DRGH_12). Lastly, several strains were covered by multiple ASVs, most probably because of the short amplicon length.

4. Discussion

Here, we examined (i) the edaphic (soil physicochemical and biological properties), environmental (drought treatment), and host factors (genotype) that might explain the variations in the soil and the endosphere communities, (ii) the microbial differences between the soil and the endosphere samples, (iii) the bacterial modules in relation to the soil properties, (iv) the effects of drought stress on the soil and the endosphere communities, and (v) the specific microbial strains that could potentially protect wheat plants against drought. First, we observed that the other environmental factors (i.e., soil regions and types) had the large explanatory power when combined together, but masked all other edaphic factors when analyzed individually, suggesting that the effect of environmental factors in the present study were mostly driven by the soil physicochemical and biological properties. To determine the biogeographical impacts on the microbiome community, an additional collection of climate variables (e.g., air temperature and humidity) and land history would be needed [40]. Soil composition is known to significantly influence both the soil and root microbial communities [41,42,68]. Previous tests on several soil properties revealed that the soil pH most strongly correlated with the community structure regardless of the studied parameters, in agreement with our results [69–71]. Additionally, we discovered that the microbiota in each soil was linked to different bacterial contributors in line with the pH of the different soil types. For example, the microbiome compositions under moderate pHs, namely ME (pH 6.16), PO (pH 7.33), and WA (pH 6.49), were strongly associated with dominant phyla. By contrast, we found that the low-abundant phyla correlated with the microbiome composition in more acidic soils (pH 5.55 and 5.71), implying that soils at these pHs provide a limited fostering environment for specific bacteria compared to soils at neutral/moderate pHs. The bacterial diversity had also previously been reported to show a curved shape along the soil pHs with a peak at neutral pHs, indicating that most bacteria grow best at neutral pHs [70]. This phenomenon could be further unraveled through evaluation of the pH resistance of different soil taxa, ultimately leading to the construction of bacterial
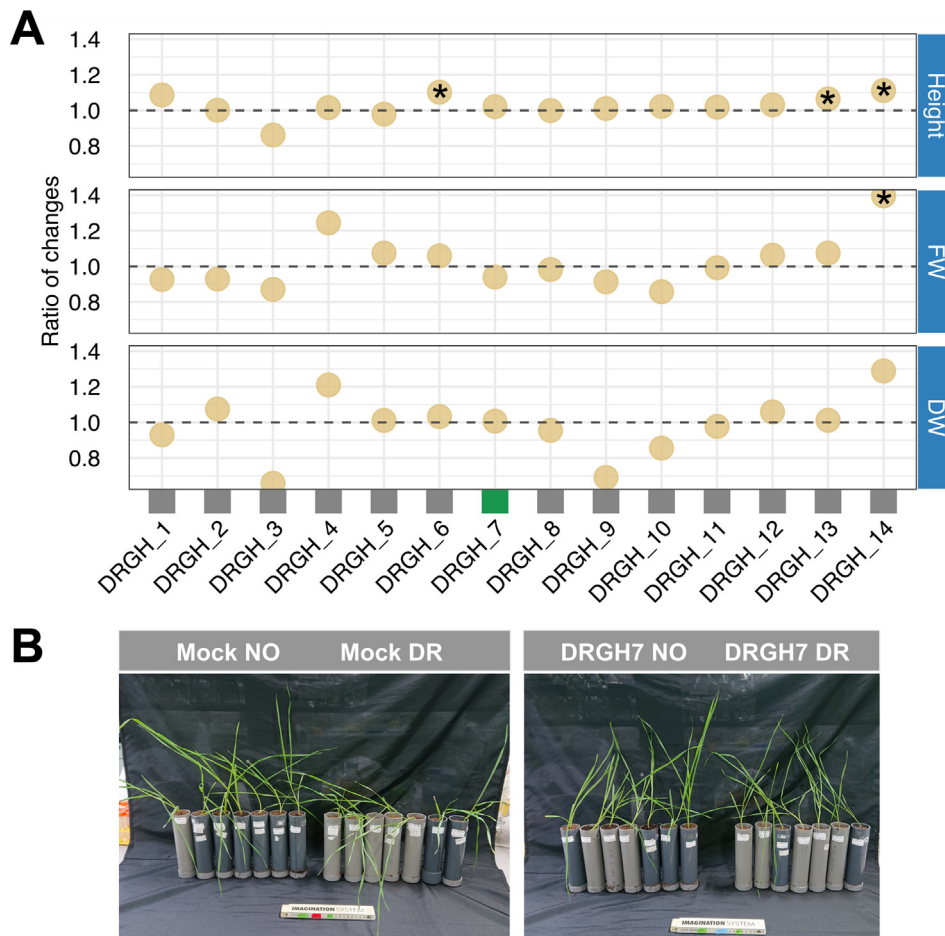
Fig. 6. Screening of *Streptomyces* isolates under drought stress. (A) Ratio of changes compared to mock-inoculated control (set to 1) plotted for plant height, shoot fresh (FW), and dry weight (DW). Green and grey squares represent plants wilting under drought treatment. * Wilcoxon rank sum test, P < 0.05. (B) Comparisons between wheat, mock-treated (left), and wheat, inoculated with DRGH strains (right) under well-watered (NO) and drought (DR) conditions. Only wheat plants upright under the drought condition upon bacterial inoculation are shown (see green squares in Fig. 6A). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

libraries that withstand different pH conditions and to the development of PGPR strains that are adapted to specific soil environments. In addition to the soil pH, we demonstrated that the microbial biomass, drought treatment, iron (soil community only), sodium (endosphere community only), and wheat genotype were associated with the community variations. In particular, the bacterial biomass had a greater effect size than the drought stress in the present study. The relationship between biomass and community diversity is not unidirectional. Under scarce resources with low biomass, the relationship between the two can be positive, whereas the presence of dominant species under high biomass will reduce the diversity [43]. Both iron and sodium have been linked previously with plant–microbe-soil interactions. Iron, an essential micronutrient for physiological and biochemical metabolisms in plants, has recently been shown to possibly protect plants against phytopathogens when scavenged by soil bacteria [44,45]. By contrast, sodium acts as a barrier in plant growth and soil microbial activity by accumulating salinity in the soil [46]. Based on these observations, the question can be addressed whether the differences in sodium and iron cause the changes in the microbial endosphere community, or whether the soil type affects the plant itself, in turn, establishing a different microbiome in the plant-root environment. Although previous research showed that genotype was a main factor for the endosphere and rhizosphere microbial communities in olive (*Olea europaea* L.) root [72], the wheat plant geno-

types were reported to have measurable, but small, effects on the root microbiota composition [47]. The extent of the impact of the plant genotype in wheat has been indicated to be approximately 2%, as confirmed in the present study.

Drought stress not only perturbs the plant phenotype, but also the microbial community structure, leading to a decrease in the community diversity [13,48–50]. Interestingly, we witnessed opposite response patterns: the community diversity significantly decreased in the soil under drought treatment, but it did not differ or rather increased in the endosphere community. The detected decrease in community diversity in the soil is most probably due to the unfavorable bacterial growth conditions induced by the drought treatment. For example, the drought treatment might create a more static environment not beneficial for bacterial growth. On the contrary, the increase in the endosphere could be a consequence of the plant's defense mechanism in an effort to retain drought-tolerant bacteria in its roots. For instance, previous research reported that enrichment of monoderm (Gram-positive bacteria) and their metabolites could promote drought tolerance [49,51]. This adaptation of the plant to drought stress might result in an active adjustment of the microbial community in the root environment. Additionally, plant exudates are known to play an important role in shaping the root microbiome by increasing the production of carbohydrates (i.e., xylose and glucose) and amino acids (i.e., proline, threonine, and asparagine), which can promote

monoderm growth [49,73,74]. Tackling this hypothetical link can give us more insights into the microbial dynamics that occur in the endosphere upon drought treatment.

By means of the WGCNA approach that detects bacterial sub-modules and their associations with host traits, we identified two modules that contained the most drought-enriched genera, several belonging to the phylum Actinobacteria, and that showed identical correlations to several soil parameters, both in the soil and in the root. These correlations were negative with soil moisture, organic carbon, Ca, and Na and were positive with nitrogen-related nutrients (total nitrogen and NO_3^--N) and dry matter. In agreement, the same modules in the soil shared the similar trends for NO_3^--N (+), Ca (-), and Na (-) with the endosphere modules, implying that these parameters are truly main soil conditions for the drought-resistant bacteria. Exchangeable Ca was reported to be one of the covariates that can explain the Actinobacterial community composition [52]. The link between nitrogen and the drought-enriched bacterial modules could be attributed to some Actinobacteria that can fix nitrogen or to carbon substrates produced by plants, promoting the growth of diazotrophic bacteria [53,75]. Our results indicate that the drought-enriched bacteria thrive when there is less moisture, Ca, and Na, but more nitrogen in the soil.

To understand potential drought-resistant mechanisms induced by the endosphere community, we investigated the functional potential (i.e., KOs) predicted by the 16S rRNA gene under drought stress. Next, the drought-enriched KOs were aligned to the bacterial modules identified using the WGCNA approach. With focus on the Orange module with the most drought-enriched genera, the strongest associations were from β-glucosidase, the osmoprotectant transport system permease protein (opuBD), and aldehyde dehydrogenase (ALDH). As previously reported, β-glucosidase plays an important role as a defensive plant protein and overexpression of the gene enhances drought tolerance [54–56]. Osmoprotectant, such as betaine, proline, and trehalose, also protect plants from drought and salinity by osmotic adjustment [57]. Specifically, ALDH provides a critical step in the proline production by catalyzing glutamate [58]. Moreover, proline, a marker for abiotic stress in plants [59], has been reported to be produced by two endogenous soil bacteria, *Streptomyces griseus* and *Streptomyces californicus*, when under salinity stress [60]. These results imply that the inferred microbial pathway related to ALDH could be induced by *Streptomyces*. However, further studies are needed to experimentally confirm the mechanisms behind the increase in drought-enriched bacteria and proline production.

Actinobacteria have been consistently detected to be enriched in soil and root environments upon drought in different crops, such as grass species, peanut (*Arachis hypogaea*) and other angiosperms [12,13,61,62] and to confer resilience against drought stress when directly inoculated into plants [63,64]. Our *in silico* analysis further validated that Actinobacteria, and more specifically the genus *Streptomyces* within the Streptomycetaceae, contained the largest proportion of drought-enriched ASVs. We further confirmed that several isolates belonging to the *Streptomyces* genus, Actinobacteria phylum, significantly improved plant height and shoot fresh weight and prevented wilting under drought stress. In spite of a robust signal from our *in silico* results indicating that *Streptomyces* increase in response to drought treatment, the *in planta* results indicated strain-specific effects, because not all isolates belonging to the selected genera induced protective effects upon drought treatment. Moreover, many of the isolates tested matched to multiple ASVs, as observed previously [76], and anticipated because of their intrinsic short sequences following a decreased specificity. The resolution at the lowest taxonomic level was not sufficient enough to distinguish individual isolates. Hence, high throughput amplicon sequencing of full-length 16S rRNA by means of new long-read sequence technologies could provide a solution to improve the pairing of bacterial isolates with microbiome data [65]. Upon the availability of such long-read sequences, a further test of strains belonging to genera (or ASVs) that are not enriched under drought stress would have to be done to evaluate the strategy of microbiome-assisted selection. In this manner, the true effects of the isolates that had been selected based on the microbiome data might be corroborated. Taking the limitations of the current sequencing approach into account, we show that amplicon sequencing data can be utilized to narrow down potential candidates for *in planta* testing.

5. Conclusion

In summary, we determined specific soil compositions that are important drivers of the microbial community assembly in both soil and wheat root environments with and without drought stress. Soil type, soil pH, microbial biomass, genotype, sodium, and iron were identified as the most important covariates associated with the soil and endosphere community variations. Under drought stress, the community richness response was opposite in soil and root endosphere, namely the diversity significantly decreased in the soil, but increased in the endosphere. Furthermore, the co-occurrence analysis revealed a cluster of drought-enriched bacteria linked with soils with low calcium and sodium levels, but high nitrogen levels. This bacterial cluster additionally presented strong associations with a drought-enriched functional potential involving stress response proteins. The phylum Actinobacteria and many of its genera/ASVs were again shown to be clearly enriched upon drought stress and potentially associated with the soil drought parameters identified in the present study. Using this *in silico* microbiome analysis as a filter, we further identified potential PGPR strains belonging to the actinobacterial genera *Streptomyces* with a protective effect against drought stress. Our findings support the efforts to increase crop yield under adverse conditions, such as drought, through the implementation of root microbiome dynamics and to provide PGPR targets with specific soil properties for further development.

CRediT authorship contribution statement

Jiyeon Si: Data curation, Formal analysis, Writing - original draft, Funding acquisition. **Emilie Froussart:** Conceptualization, Data curation, Writing - original draft. **Tom Viaene:** Conceptualization, Resources, Writing - original draft. Jorge F. Vázquez-Castellano: Formal analysis, Writing - review & editing. **Kelly Hamonts:** Resources, Writing - review & editing. **Lin Tang:** Conceptualization, Writing - review & editing. **Stien Beirinckx:** Conceptualization, Writing - review & editing. **Annick De Keyser:** Resources. **Tibby Deckers:** Resources. **Fien Amery:** Resources. **Steven Vandenabeele:** Conceptualization, Writing - review & editing. **Jeroen Raes:** Conceptualization, Funding acquisition, Supervision. **Sofie Goormachtig:** Conceptualization, Writing - original draft, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability Statement

The raw sequence reads were deposited and are available for download at the https://www.ebi.ac.uk/ena/browser/home with project number PRJEB40100.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2021.07.027.

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