Plant Soil (2018) 422:35–49
DOI 10.1007/s11104-016-3113-9

REGULAR ARTICLE

The *Cucurbita pepo* seed microbiome: genotype-specific composition and implications for breeding

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Received: 31 August 2016 / Accepted: 15 November 2016 / Published online: 25 November 2016

Abstract
Background and aims Plant breeding activities shape the rhizosphere microbiome but less is known about the relationship of both with the seed microbiome. We analyzed the composition of bacterial communities of seeds and rhizospheres of Styrian oil pumpkin genotypes in comparison to bulk soil to elucidate specific microbial signatures to support a concept involving plant-microbe interactions in breeding strategies.
Methods The seed and rhizosphere microbiomes of 14 genotypes of oilseed pumpkin and relatives were analyzed using a 16S rRNA gene amplicon sequencing approach, which was assessed by bioinformatics and statistical methods.
Results All analyzed microhabitats were characterized by diverse bacterial communities, but the relative proportions of phyla and the overall diversity was different. Seed microbiomes were characterized by the lowest diversity and dominant members of *Enterobacteriaceae* including potential pathogens (*Erwinia, Pectobacterium).* Potential plant-beneficial bacteria like *Lysobacter*, *Paenibacillus* and *Lactococcus* contributed to the microbial communities in significant abundances. Interestingly, strong genotype-specific microbiomes were detected for seeds but not for the rhizospheres.
Conclusions Our study indicates a strong impact of the *Cucurbita pepo* genotype on the composition of the seed microbiome. This should be considered in breeding of new cultivars that are more capable of exploiting beneficial indigenous microbial communities.

Keywords Plant-microbe interactions · *Cucurbitaceae* · Pumpkin · Bacterial diversity · 16S rRNA gene amplicon sequencing · *Enterobacteriaceae*

Introduction

Microbial communities have central roles for plant development and health throughout the entire life cycle (Mendes et al. 2011; Philippot et al. 2013). This knowledge has been a revolutionary advance in biological sciences, also directing plant research towards a more holistic view (Berg et al. 2016). Since more than a century, diversity and function of the rhizosphere microbiome was intensively studied and the impact of the plant genotype and soil quality determined (Hiltner 1904; Smalla et al. 2001; Berg and Smalla 2009; Lundberg et al. 2012). Recent studies on crop cultivars revealed that breeding shapes the composition of the root-associated bacterial communities including the antagonistic potential towards pathogens (Peiffer and Ley 2013; Bouffaud et al. 2014; Cardinale et al. 2015). In the

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Responsible Editor: Philippe Simoneau.

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past, mainly seed-borne pathogens were studied but recently the influence of the whole seed microbiome on plant health has gained more interest (Aleklett and Hart 2013; Barret et al. 2015). Seeds are of particular interest as microbial carriers because they are involved in the transmission of both potential beneficial and pathogenic microorganisms from one generation to another (Johnston-Monje et al. 2016). Breeding plants for beneficial plant-microbe interactions is an emerging field mainly focusing on below ground interactions in the rhizosphere (reviewed in Wissuwa et al. 2009; Bakker et al. 2012); however the impact of the seed microbiome is completely unclear.

Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) is a cultural heritage of the Austro-Hungarian Empire and of international importance today. The seeds are used for the production of a unique, dark-green seed oil, which is traditionally consumed in Austria, and is increasingly popular in gourmet cuisines worldwide. In 2016, the acreage of the Styrian oil pumpkin reached a peak level with 39,450 ha in Austria, and the market demand for this high-value crop continues to rise. New growing areas have been mainly established in China but also in the US. The morphological structure of *C. pepo* seeds comprises a root-hypocotyl embryo, two distinct photosynthetic cotyledons, a thin endosperm, remains of the nucellus and a seed coat with five layers. Styrian oil pumpkin emerged from a natural mutation and is lacking a lignification of the seed coat resulting in a high susceptibility to various fungal and bacterial pathogens during seed germination (Heinisch and Ruthenberg 1950). Thus, commercially available Styrian oil pumpkin seeds are by default treated with chemical strippers; mainly synthetic fungicides or copper-based products. Sowing of untreated seeds generally results in a drastic reduction of germination rates or germination fails totally, if weather or soil conditions are unfavorable after sowing. A disease responsible for high yield losses is the fruit rot caused by the consortium of *Didymella bryoniae* and *Pectobacterium carotovorum* (syn. *Erwinia carotovora*) (Grube et al. 2011) or *Erwinia atrosepticum*. Leaves of adult plants can be infested by fungi such as *Didymella bryoniae* or *Phyllosticta cucurbitacearum* (Bedlan 2012) and by bacterial pathogens like *Xanthomonas campestris* pv. *cucurbitae, Pseudomonas syringae* or *P. viridiflava*. Strong cultivar-specificity regarding the susceptibility to fruit rot was shown in evaluations of registered oil pumpkin cultivars (AGES 2016; Winkler et al. 2008). A high genotype-specificity has been widely shown for the interactions of plants with pathogens (Neupane et al. 2015; Bruns et al. 2012; Rubiales and Niks 1996) and thus breeding for resistances against pathogens is common practice (Niks et al. 2011; Pachner et al. 2015; Roane 1973; Ashkani et al. 2015). Conversely, genotype-specific beneficial plant-microbe associations have not been considered in breeding strategies thus far. Our hypothesis was that pumpkin seeds contain a genotype-specific microbiome, which consists of a core subset of the plant-associated microbiome with mainly plant-beneficial traits.

The objective of this study was a comprehensive microbiome analysis based on a 16S rRNA gene sequencing approach targeting the bacterial and parts of the archaeal diversity of 14 *C. pepo* genotypes. Those included one open-pollinated cultivar, three hybrids and their pedigree components (four inbred lines) and five segregating lines of the Styrian oil pumpkin as well as a zucchini hybrid. The study should be rated as a first step towards gaining a deeper understanding of genotype-dependent differences in plant-microbe interactions of the Styrian oil pumpkin. The overall aim is the development of a concept to (re-)integrate beneficial plant-microbe interactions into the plant breeding activities of the Styrian oil pumpkin.

Materials & methods

Pumpkin genotypes

Upon selection of the *C. pepo* genotypes for analysis, the focus was toward coverage of cultivars with a high market share (three-way cross hybrids ‘GL Opal’ and ‘GL Rustikal’) including their pedigree components (inbred Line A – D and the single cross hybrid ‘Gleisdorfer Diamant’ (‘Gl. Diamant’)). An open-pollinated cultivar frequently used in organic agricultural systems, ‘GL Classic’, and six other cultigens bred in countries other than Austria were included to broaden the spectrum of genotypes (Table 1). The geographic origin records the country in which a genotype was selected or bred. With the exception of the single cross zucchini hybrid Naxos, the seeds used for amplicon sequencing approaches were harvested from plants grown on three different field sites near Gleisdorf (province of Styria, Austria). Post-harvest processing of those seeds was performed according to the standard

procedures of the Saatzucht Gleisdorf GmbH breeding station.

Sampling of seed, rhizosphere and soil replicates

For the seed microbiome analysis, 40 seeds of each genotype were washed five times for one minute with 50 mL sterile deionized water and soaked in 25 mL sterile deionized water for 4 h on a rotary shaker at 100 rpm. Subsequently, the seeds were divided into four replicates of 10 seeds each and were ground with a pestle in 10 mL 0.85% NaCl in sterile bags (Nasco Whirl-Pak®). For each replicate, a 3 mL suspension was pelleted by 20 min centrifugation at 4°C and 13,500 g.

For the rhizosphere microbiome analysis, 40 seeds per genotype of the same seed lots used for seed microbiome analysis were coated with 0.3 g of the fungicide Maxim® XL (Syngenta) and split into four replicates. Seeds were sown at a field site near the breeding station of Saatzucht Gleisdorf GmbH in randomized plots (47°06′57.3″N, 15°42′31.3″ E). The soil of the field site is described as gleyed loose brown earth, loamy silt, and cover loams on a quaternary terrace deficient in lime, with a pH-value of 6.5. In parallel 40 seeds per genotype were sown without fungicide coating on the same field site. As only 2.7% of those plants emerged, the rhizosphere samples had to be taken from plants grown from fungicide treated seeds (93.1% germination rate): one month after sowing, rhizosphere material from four randomly chosen plants per plot was sampled and pooled. Additionally, four bulk soil samples were taken from random places at the field site. Five to 7 g of each rhizosphere and soil replicate were suspended in 50 mL 0.85% NaCl and homogenized by a 3 min bag mixer (stomacher) treatment, then 4 mL of the homogenized solution were pelleted as described above.

DNA extraction and amplicon sequencing

The DNA extraction was performed using a modified protocol of the FastDNA™ SPIN Kit for Soil (MP Biomedicals). The variable region 4 of the 16S rRNA gene was amplified with 515f (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806r (5′-GGACTACHVGGGTWTCTAAT-3′) primers extended by individual barcodes. For PCR amplification, a modified protocol of Lundberg et al. (2013) including synthetic peptide nucleic acid PCR clamps (PNAs) for blocking the amplification of mitochondrial and plastid 16S rRNA gene sequences of plants was applied. Three independent PCR amplifications were performed per replicate sample. The triplicate amplification products were pooled and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) protocol. The PCR products were adjusted volumetrically to reach equimolarity of each sample in one common pool for 16S rDNA sequencing. Amplicon sequencing was performed with the Illumina MiSeq V2 sequencing platform (2 × 150 bp paired-end) by GATC Biotech (Germany).

Bioinformatic and statistical analyses

Following de-multiplexing of raw reads and clipping of sequencing adapters, read pairs were joined and sorted according to sample-specific barcodes. Joint reads were further analyzed with the open-source bioinformatics pipeline QIIME 1.9.1 (Caporaso et al. 2010a). First, reads were quality (Phred score ≥ 20) and length (290–300 bp) filtered, and primer and barcodes flanking the reads were clipped. Chimeric sequences were removed by means of the de novo UCHIME method (Edgar 2010; Edgar et al. 2011). Remaining sequences were clustered into operational taxonomic units (OTUs) at a 97% similarity level using the de novo UCLUST clustering method with default parameters (Edgar 2010). The most abundant representative read per OTU was picked and taxonomically assigned using RDP Classifier 2.2. (Wang et al. 2007) based on the reference database Greengenes release gg_13_8_99 (DeSantis et al. 2006). The alignment of reads was performed using PyNAST (Caporaso et al. 2010b). Additionally, an approximately-maximum-likelihood phylogenetic tree using FastTree 2.1.3. (Price et al. 2010) was generated.

Each replicate was comprised of 11,245 to 276,132 sequences after initial data processing. Prior to statistical analyses, the mitochondrial (ranging from 0.6% to 32.9% per replicate) and plastid (0.3% to 6.8%) sequences with plant origin and unassigned OTUs were excluded by filtering the OTU table. Additionally, out of four replicates per sample, the replicate with the lowest read number was discarded. As the remaining replicates comprised of 3758 to 256,248 sequences, the subsequent analyses were performed after normalizing the sequence number per replicate to 3758.

Table 1 Characteristics of *Cucurbita pepo* genotypes selected for the microbiome analysis

Denomination	Category*	Pedigree	Geographic origin	Field site origin of seeds/harvest year
Line A	Inbred line (nl)	–	Austria	47°06′48.4″N, 15°42′06.9″E/2014
Line B	Inbred line (nl)	–	Austria	47°06′48.4″N, 15°42′06.9″E/2014
Line C	Inbred line (nl)	–	Austria	47°07′01.8″N, 15°42′24.8″E/2013
Line D	Inbred line (nl)	–	Austria	47°06′48.4″N, 15°42′06.9″E/2014
Gl. Diamant	Single cross hybrid (nl)	Line A × Line B	Austria	47°07′01.8″N, 15°42′24.8″E/2014
GL Opal	Three-way cross hybrid (nl)	Gl. Diamant × Line C	Austria	47°08′04.9″N 15°40′58.4″E/2014
GL Rustikal	Three-way cross hybrid (nl)	Gl. Diamant × Line D	Austria	47°07′01.8″N, 15°42′24.8″E/2014
GL Classic	Open-pollinated cultivar (nl)	–	Austria	47°06′48.4″N, 15°42′06.9″E/2014
Naxos	Single cross zucchini hybrid (l)	Unknown	Netherlands	Unknown/unknown
Line E	Segregating line (nl)	–	Germany	47°07′01.8″N, 15°42′24.8″E/2014
Line F	Segregating line (l)	–	Slovenia	47°07′01.8″N, 15°42′24.8″E/2014
Line G	Segregating line (nl)	–	Slovenia	47°07′01.8″N, 15°42′24.8″E/2014
Line H	Segregating line (l)	–	China	47°07′01.8″N, 15°42′24.8″E/2014
Line I	Segregating line (l)	–	China	47°07′01.8″N, 15°42′24.8″E/2014

**nl* no lignification of the seed coat, *l* lignification of the seed coat

Statistical analyses to calculate significance of differences in diversity indices were performed using the non-parametric Kruskal-Wallis Rank Sum Test (Hollander and Wolfe 1973) and the Pairwise Test for Multiple Comparisons of Mean Rank Sums (Nemenyi-Test) (Sachs 1997) implemented in the open source data analysis software RStudio (RStudio Team 2015). Non-parametric analyses of similarities (ANOSIM) were calculated according to Fierer et al. (2010) and Clarke (1993). A non-metric multidimensional scaling (NMDS) analysis was performed using the open source data analysis software RStudio and the function metaMDS {vegan} (Faith et al. 1987; Minchin 1987) with calculation of the distance matrix based on a Bray-Curtis algorithm.

Results

Microbial communities associated with seed, rhizosphere and soil

All analyzed microhabitats were characterized by a high diversity of certain phyla but the relative proportions were different (Fig. 1). *Proteobacteria* predominated seed (83%), rhizosphere (41%) and soil (24%) microbiomes and considerable proportions of the phyla *Firmicutes* (11%, 8% and 6%) and *Actinobacteria* (2%, 17% and 15%) were found in all habitats. *Thaumarchaeota* as well as *Acidobacteria, Bacteroidetes, Chloroflexi, Nitrospirae, Gemmatimonadetes, Planctomycetes,* and *Verrucomicrobia* contributed to the microbiomes of the rhizosphere and soil and only to minor degree to the seed microbiome.

OTUs representing the core microbiome were calculated separately for each of the habitats seed, rhizosphere and soil and then summarized (Fig. 2). Differences in the relative abundance of those OTUs between the core microbiomes exist for several *Nitrososphaeria, Acidobacteria, Chloracidobacteria* and *Chloroflexi* OTUs, which occurred in a higher proportion in the soil microbiome compared to the rhizosphere and seed microbiomes, whereas several *Bacilli, Actinobacteria, Saprospirae, Alpha-, Beta-* and parts of the *Gammaproteobacteria* OTUs occurred to a greater degree in the rhizosphere microbiome in comparison to the soil and seed microbiomes. The seed core microbiome was dominated by high abundances of eight *Gammaproteobacteria* (seven *Enterobacteriaceae* and one *Pseudomonadaceae*) and two *Bacilli* (one *Lactococcus* and one *Exiguobacterium*) OTUs.

OTU distribution and diversity analyses

The comparison of the seed and rhizosphere core microbiomes with the bulk soil microbiome shows that seeds and rhizosphere shared only 10.5% of the total OTUs including OTUs from soil, whereas the

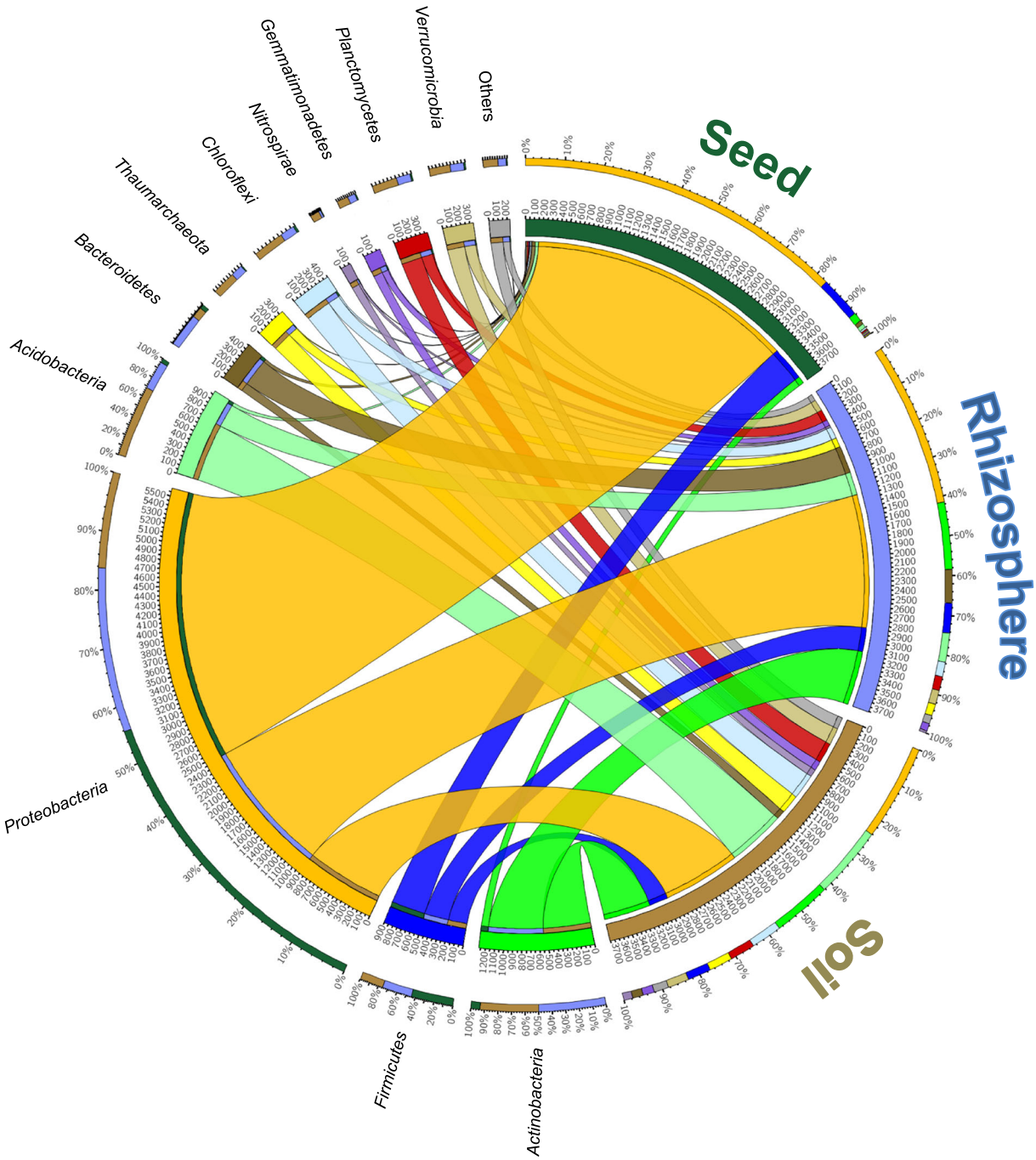
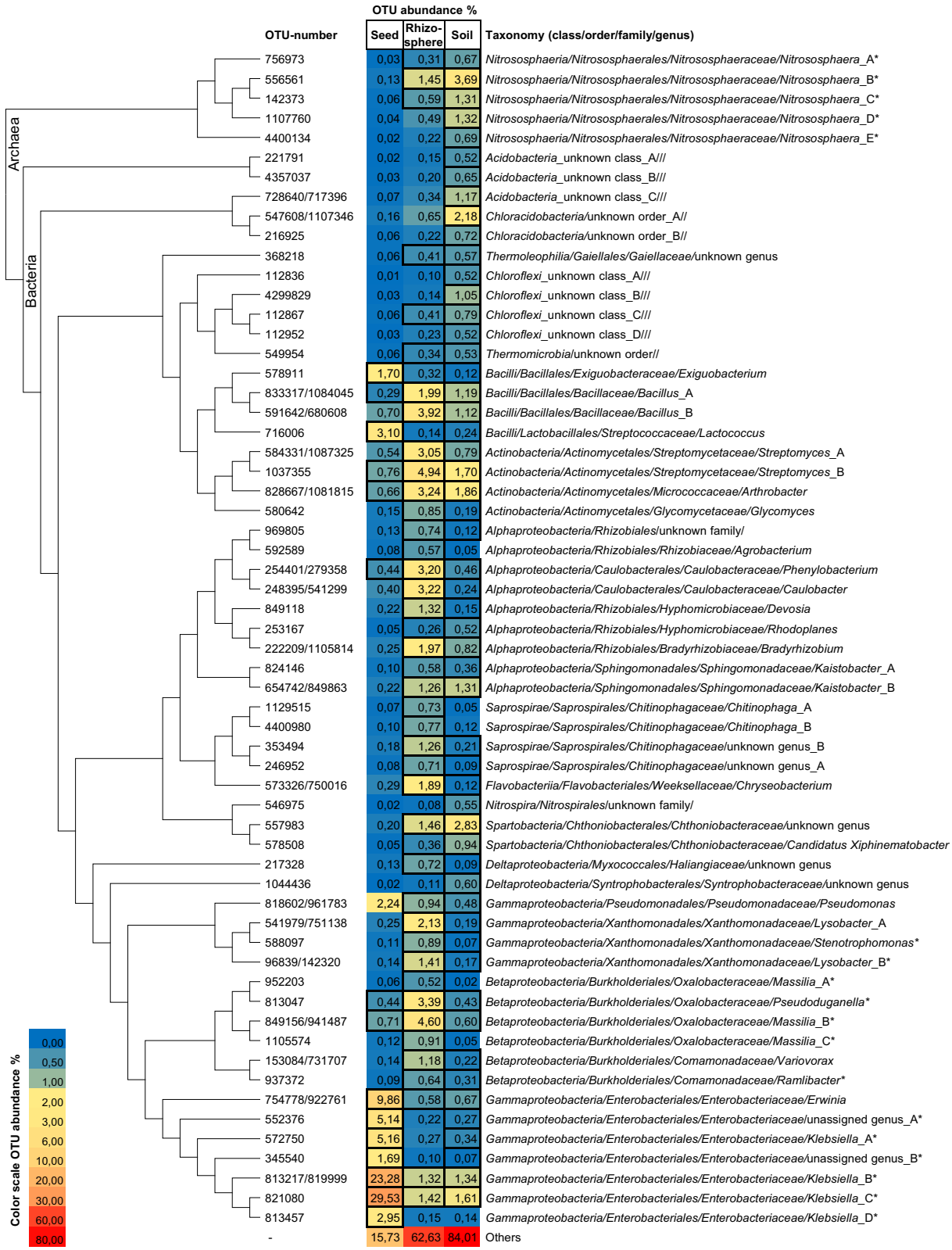


Fig. 1 Circular representation of the proportional structure of bacterial communities at phylum level associated with rhizosphere and seeds of *C. pepo* genotypes as well as soil (outer circle). Taxa with a proportion lower than 1.5% in all three habitats are summarized as 'Others'. Values within the inner circle indicate the number of reads of a phylum within the normalized dataset. The graphic was built using the open-source software Circos (Krzywinski et al. 2009)

rhizosphere and the bulk soil shared 32.6% of those OTUs (Fig. 3a). Within the rhizosphere OTUs 16.8% were conserved in seeds and soil as well, 4.5% were derived solely from the seeds and 49.6% solely from the soil. Apparently, the seed microbiome has a smaller influence on the rhizosphere communities than the soil


OTU abundance %
OTU-number
Seed
Rhizo-sphere
Soil
Taxonomy (class/order/family/genus)
Archaea
Bacteria
756973 0,03 0,31 0,67 Nitrososphaeria/Nitrososphaerales/Nitrososphaeraceae/Nitrososphaera_A*
556561 0,13 1,45 3,69 Nitrososphaeria/Nitrososphaerales/Nitrososphaeraceae/Nitrososphaera_B*
142373 0,06 0,59 1,31 Nitrososphaeria/Nitrososphaerales/Nitrososphaeraceae/Nitrososphaera_C*
1107760 0,04 0,49 1,32 Nitrososphaeria/Nitrososphaerales/Nitrososphaeraceae/Nitrososphaera_D*
4400134 0,02 0,22 0,69 Nitrososphaeria/Nitrososphaerales/Nitrososphaeraceae/Nitrososphaera_E*
221791 0,02 0,15 0,52 Acidobacteria_unknown class_A///
4357037 0,03 0,20 0,65 Acidobacteria_unknown class_B///
728640/717396 0,07 0,34 1,17 Acidobacteria_unknown class_C///
547608/1107346 0,16 0,65 2,18 Chloracidobacteria/unknown order_A//
216925 0,06 0,22 0,72 Chloracidobacteria/unknown order_B//
368218 0,06 0,41 0,57 Thermoleophilia/Gaiellales/Gaiellaceae/unknown genus
112836 0,01 0,10 0,52 Chloroflexi_unknown class_A///
4299829 0,03 0,14 1,05 Chloroflexi_unknown class_B///
112867 0,06 0,41 0,79 Chloroflexi_unknown class_C///
112952 0,03 0,23 0,52 Chloroflexi_unknown class_D///
549954 0,06 0,34 0,53 Thermomicrobia/unknown order//
578911 1,70 0,32 0,12 Bacilli/Bacillales/Exiguobacteraceae/Exiguobacterium
833317/1084045 0,29 1,99 1,19 Bacilli/Bacillales/Bacillaceae/Bacillus_A
591642/680608 0,70 3,92 1,12 Bacilli/Bacillales/Bacillaceae/Bacillus_B
716006 3,10 0,14 0,24 Bacilli/Lactobacillales/Streptococcaceae/Lactococcus
584331/1087325 0,54 3,05 0,79 Actinobacteria/Actinomycetales/Streptomycetaceae/Streptomyces_A
1037355 0,76 4,94 1,70 Actinobacteria/Actinomycetales/Streptomycetaceae/Streptomyces_B
828667/1081815 0,66 3,24 1,86 Actinobacteria/Actinomycetales/Micrococcaceae/Arthrobacter
580642 0,15 0,85 0,19 Actinobacteria/Actinomycetales/Glycomycetaceae/Glycomyces
969805 0,13 0,74 0,12 Alphaproteobacteria/Rhizobiales/unknown family/
592589 0,08 0,57 0,05 Alphaproteobacteria/Rhizobiales/Rhizobiaceae/Agrobacterium
254401/279358 0,44 3,20 0,46 Alphaproteobacteria/Caulobacterales/Caulobacteraceae/Phenylobacterium
248395/541299 0,40 3,22 0,24 Alphaproteobacteria/Caulobacterales/Caulobacteraceae/Caulobacter
849118 0,22 1,32 0,15 Alphaproteobacteria/Rhizobiales/Hyphomicrobiaceae/Devosia
253167 0,05 0,26 0,52 Alphaproteobacteria/Rhizobiales/Hyphomicrobiaceae/Rhodoplanes
222209/1105814 0,25 1,97 0,82 Alphaproteobacteria/Rhizobiales/Bradyrhizobiaceae/Bradyrhizobium
824146 0,10 0,58 0,36 Alphaproteobacteria/Sphingomonadales/Sphingomonadaceae/Kaistobacter_A
654742/849863 0,22 1,26 1,31 Alphaproteobacteria/Sphingomonadales/Sphingomonadaceae/Kaistobacter_B
1129515 0,07 0,73 0,05 Saprospirae/Saprospirales/Chitinophagaceae/Chitinophaga_A
4400980 0,10 0,77 0,12 Saprospirae/Saprospirales/Chitinophagaceae/Chitinophaga_B
353494 0,18 1,26 0,21 Saprospirae/Saprospirales/Chitinophagaceae/unknown genus_B
246952 0,08 0,71 0,09 Saprospirae/Saprospirales/Chitinophagaceae/unknown genus_A
573326/750016 0,29 1,89 0,12 Flavobacteriia/Flavobacteriales/Weeksellaceae/Chryseobacterium
546975 0,02 0,08 0,55 Nitrospira/Nitrospirales/unknown family/
557983 0,20 1,46 2,83 Spartobacteria/Chthoniobacterales/Chthoniobacteraceae/unknown genus
578508 0,05 0,36 0,94 Spartobacteria/Chthoniobacterales/Chthoniobacteraceae/Candidatus Xiphinematobacter
217328 0,13 0,72 0,09 Deltaproteobacteria/Myxococcales/Haliangiaceae/unknown genus
1044436 0,02 0,11 0,60 Deltaproteobacteria/Syntrophobacterales/Syntrophobacteraceae/unknown genus
818602/961783 2,24 0,94 0,48 Gammaproteobacteria/Pseudomonadales/Pseudomonadaceae/Pseudomonas
541979/751138 0,25 2,13 0,19 Gammaproteobacteria/Xanthomonadales/Xanthomonadaceae/Lysobacter_A
588097 0,11 0,89 0,07 Gammaproteobacteria/Xanthomonadales/Xanthomonadaceae/Stenotrophomonas*
96839/142320 0,14 1,41 0,17 Gammaproteobacteria/Xanthomonadales/Xanthomonadaceae/Lysobacter_B*
952203 0,06 0,52 0,02 Betaproteobacteria/Burkholderiales/Oxalobacteraceae/Massilia_A*
813047 0,44 3,39 0,43 Betaproteobacteria/Burkholderiales/Oxalobacteraceae/Pseudoduganella*
849156/941487 0,71 4,60 0,60 Betaproteobacteria/Burkholderiales/Oxalobacteraceae/Massilia_B*
1105574 0,12 0,91 0,05 Betaproteobacteria/Burkholderiales/Oxalobacteraceae/Massilia_C*
153084/731707 0,14 1,18 0,22 Betaproteobacteria/Burkholderiales/Comamonadaceae/Variovorax
937372 0,09 0,64 0,31 Betaproteobacteria/Burkholderiales/Comamonadaceae/Ramlibacter*
754778/922761 9,86 0,58 0,67 Gammaproteobacteria/Enterobacteriales/Enterobacteriaceae/Erwinia
552376 5,14 0,22 0,27 Gammaproteobacteria/Enterobacteriales/Enterobacteriaceae/unassigned genus_A*
572750 5,16 0,27 0,34 Gammaproteobacteria/Enterobacteriales/Enterobacteriaceae/Klebsiella_A*
345540 1,69 0,10 0,07 Gammaproteobacteria/Enterobacteriales/Enterobacteriaceae/unassigned genus_B*
813217/819999 23,28 1,32 1,34 Gammaproteobacteria/Enterobacteriales/Enterobacteriaceae/Klebsiella_B*
821080 29,53 1,42 1,61 Gammaproteobacteria/Enterobacteriales/Enterobacteriaceae/Klebsiella_C*
813457 2,95 0,15 0,14 Gammaproteobacteria/Enterobacteriales/Enterobacteriaceae/Klebsiella_D*
- 15,73 62,63 84,01 Others
Color scale OTU abundance %
0,00
0,50
1,00
2,00
3,00
6,00
10,00
20,00
30,00
60,00
80,00

◀ **Fig. 2** Summary of the core microbial communities represented by 16S rRNA gene sequences in the seed and rhizosphere of *C. pepo* genotypes as well as in the bulk soil. Relative abundance values of taxa belonging to the core of a habitat are framed, while other abundance values are not belonging to the core in the respective habitat. OTUs with abundance lower than 0.5% in all core microbiomes are summarized as 'Others'. Taxa marked with asterisks were complemented with additional taxonomic information from NCBI database, while the other denominations are from Greengenes database. The phylogenetic tree using the representative sequences of the OTUs was calculated with the NCBI tree method fast minimum evolution (max sequence difference 0.75) and illustrated in MEGA7

microbiome. 29.0% of the rhizosphere OTUs were unique. Some of those OTUs could have been derived from the seed testa, but were removed due to the washing and soaking procedure for seed microbiome analysis. This probably released a number of microorganisms to the washing suspension. Another source of inoculum could have been rare soil bacteria that were below the detection level in the soil microbiome analysis but have been enriched in the rhizosphere due to the rhizosphere effect. A detailed analysis of the contribution of *Enterobacteriaceae* to the communities revealed that major proportions of those OTUs observed were unique for seeds, whereas in rhizosphere and soil no unique *Enterobacteriaceae* were detected (Fig. 3b). The same trends were observed when the analyses were calculated on *C. pepo* genotype level: the family of *Enterobacteriaceae* was strongly associated with the seed as habitat.

Alpha diversity measures Chao1 and Shannon indices revealed that the species richness in the rhizosphere was significantly higher than in the seeds (calculated with the QIIME script alpha_diversity.py; paired t-test, significance level $\alpha = 0.01$, p values = 0.003), whereas the richness in the soil was significantly higher than in the rhizosphere. The Heip index (calculated with the QIIME script alpha_diversity.py) indicated that the evenness in the seeds was considerably lower than in the rhizosphere and soil, meaning that the relative abundance of taxa was not evenly distributed. The seed microbiomes of the genotypes Line E, 'Gleisdorfer Diamant' and Line G showed a higher alpha diversity (Shannon diversity index H′ of 8.6, 7.9 and 7.1, respectively and Heip evenness index E′ of 0.29, 0.17 and 0.16, respectively) than the other investigated genotypes. In contrast to the rhizosphere, differences in alpha diversity among the seeds of several genotypes were significant (Table 2).

Genotype specific colonization patterns of seeds

The microbial seed communities of all *C. pepo* genotypes were dominated by *Proteobacteria* with 83% on average, 64% belonged to the family of *Enterobacteriaceae*. Genotype-specific colonization patterns of the seeds were evident in the open-pollinated cultivar 'GL Classic' and in the inbred Line D (Fig. 4). In those genotypes, the genus *Erwinia* was part of the microbiome with a relative abundance of 38% and 33% respectively. A detailed analysis of the sequences clustered in *Erwinia* genera revealed that they contained sequences of the important pathogen *Pectobacterium carotovorum* (syn. *Erwinia carotovora*). The genus *Pseudomonas* was present in all genotypes but was enriched in four out of six cultivars bred in other countries than Austria. *Firmicutes* were enriched in the two three-way cross hybrids 'GL Opal' (19%) and 'GL Rustikal' (36%), as well as in the two inbred Lines B and D and the segregating Line F. Within the *Firmicutes*, the genus *Lactococcus* was more abundant in four out of eight cultivars bred in the province of Styria (Austria) and only low abundant in cultivars bred in other countries. The genus *Acinetobacter* was more abundant in the segregating lines, especially in Line H with 11%. *Actinobacteria* were observed to a higher extent in Line E, Line G and 'Gleisdorfer Diamant'. These three genotypes had similar communities which were more diverse than those of the other genotypes, congruent with the results of the calculated diversity indices.

No specific patterns concerning the field site origin of studied seeds were observed. According to the non-parametric analysis of similarities the genotype ($R = 0.527$, p value = 0.001) has greater influence on the bacterial community composition than the field origin of the seeds ($R = 0.181$, p value = 0.008). While only 21 OTUs showed significantly different abundances among the rhizosphere (Kruskal-Wallis test, $\alpha = 0.01$), 121 OTUs differed significantly among the seed microbiomes of the 14 genotypes. Six OTUs showed significantly different degrees of abundancy in both habitats. These OTUs were assigned to the taxa of *Exiguobacterium*, *Chthoniobacteraceae, Nitrospirales, Xanthomonadaceae* and *Bacillus,* according to the Greengenes database. In order to visualize the beta diversity and the relationships of seed associated bacterial taxa with significant different abundances among the genotypes, a NMDS analysis of the 14 *C. pepo* genotypes was performed based on a community-by-species matrix comprising of 121 significantly different OTUs (Fig. 5).

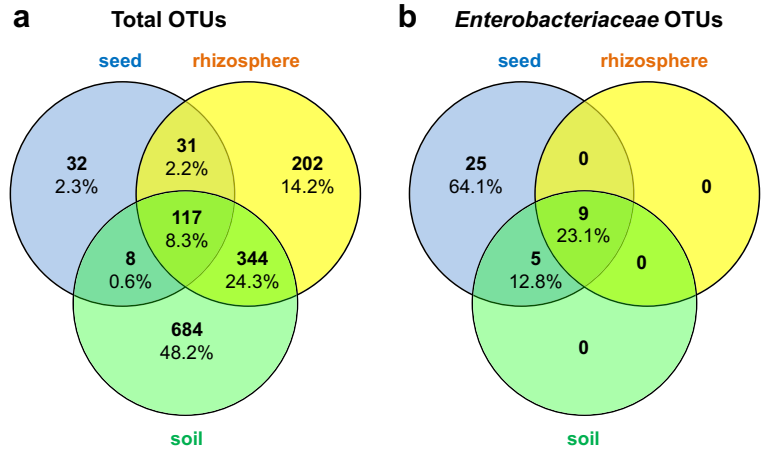


Fig. 3 OTU distribution within the habitats: **a** total OTUs and **b** *Enterobacteriaceae* OTUs in seed and rhizosphere of *C. pepo* genotypes as well as soil. Fraction of samples that OTU was observed in to be considered as 'core': 50%; the proportions are not drawn to scale

Altogether, 53 of the 121 different abundant seed-associated OTUs referred to the phylum of *Proteobacteria*, wherein 12 belonged to the family of *Enterobacteriaceae*, of which three were further assigned to the genus *Erwinia*. Higher abundances of *Pseudomonas viridiflava* were observed in the seed microbiomes of Line F and Line I as well as in the open-pollinated 'GL Classic'. Furthermore, the abundances of *Lysobacter* and *Paenibacillus* were significantly different among the seed microbiomes of the genotypes investigated. Within this analysis, the phylum *Firmicutes* comprised of 23 OTUs, wherein 11 OTUs

Table 2 Bacterial species richness, evenness and coverage in rhizosphere (R) and seed (S) of different *C. pepo* genotypes and of soil

Denomination	Shannon diversity index (H′)[1]		Heip evenness index ($E'_{1:0}$)[2]		Chao1 diversity index (OTU no.)[3]		Observed OTUs		Coverage[4]	
	R	S	R	S	R	S	R	S	R	S
Line A	9.6	3.7^{a}	0.48	0.04^{a,b}	4634	1,326^{a}	1592	334^{a}	0.34	0.25^{a}
Line B	9.3	4.1^{a,b}	0.44	0.03^{a}	3971	2,081^{a,b}	1472	468^{a,b}	0.37	0.22^{a}
Line C	9.5	4.6^{a,b}	0.47	0.04^{a,b}	4168	2,650^{a,b}	1578	565^{a,b}	0.38	0.21^{a}
Line D	9.4	4.9^{a,b}	0.45	0.05^{a,b}	4369	2,936^{a,b}	1534	613^{a,b}	0.35	0.21^{a}
Gl. Diamant	9.5	7.9^{b}	0.46	0.17^{a,b}	4819	5,651^{b}	1596	1,399^{b}	0.33	0.25^{a}
GL Opal	9.3	5.1^{a,b}	0.42	0.06^{a,b}	4772	2,017^{a,b}	1499	549^{a,b}	0.31	0.27^{a}
GL Rustikal	9.5	4.4^{a,b}	0.46	0.05^{a,b}	4642	1,646^{a,b}	1543	412^{a,b}	0.33	0.25^{a}
GL Classic	9.0	4.4^{a,b}	0.39	0.05^{a,b}	3823	1,602^{a,b}	1362	431^{a,b}	0.36	0.27^{a}
Naxos	9.3	4.5^{a,b}	0.43	0.04^{a,b}	4105	3,144^{a,b}	1444	561^{a,b}	0.35	0.18^{a}
Line E	9.1	8.6^{b}	0.41	0.29^{b}	3704	4,019^{a,b}	1395	1,324^{b}	0.38	0.33^{a}
Line F	9.3	4.7^{a,b}	0.44	0.05^{a,b}	3833	1,985^{a,b}	1448	481^{a,b}	0.38	0.24^{a}
Line G	9.4	7.1^{a,b}	0.46	0.16^{a,b}	4229	3,821^{a,b}	1509	1,124^{b}	0.36	0.29^{a}
Line H	9.1	4.7^{a,b}	0.40	0.05^{a,b}	3889	1,858^{a,b}	1366	498^{a,b}	0.35	0.27^{a}
Line I	9.3	5.9^{a,b}	0.43	0.08^{a,b}	4015	2,573^{a,b}	1450	739^{a,b}	0.36	0.29^{a}
Average	9.3	5.3^{a,b}	0.44	0.08^{a,b}	4212	2,665^{a,b}	1485	678^{a,b}	0.35	0.25^{a}
Soil	10.2		0.61		5116		1900		0.37	

[1] estimation of species diversity (a higher number indicates a higher diversity); [2] distribution of individuals over OTUs (tends to 0 as the evenness decreases in species-poor communities, tends to 1 as the individuals are increasingly distributed equally in communities); [3] non-parametric richness estimator; [4] ratio of observed OTU number to estimated OTU number; [a,b] different alphabetic characters indicate statistic significant differences

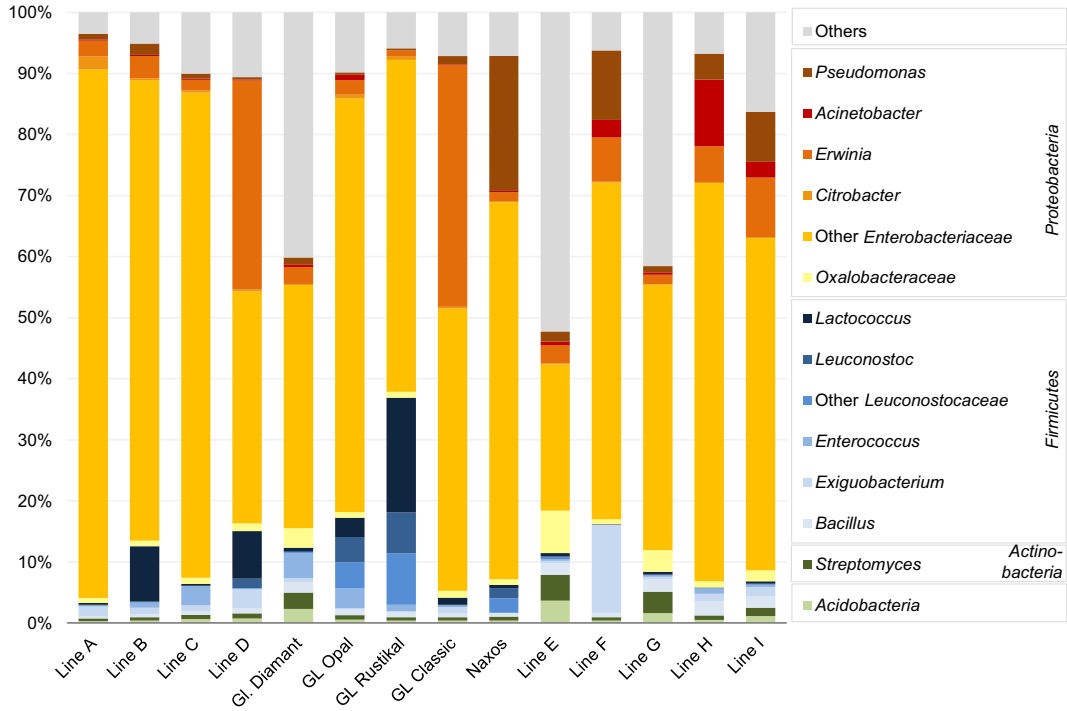


Fig. 4 Structure of bacterial taxa within seeds of *C. pepo* genotypes with a proportion higher than 2% in at least one genotype. Taxa with lower proportions are summarized as 'Others'

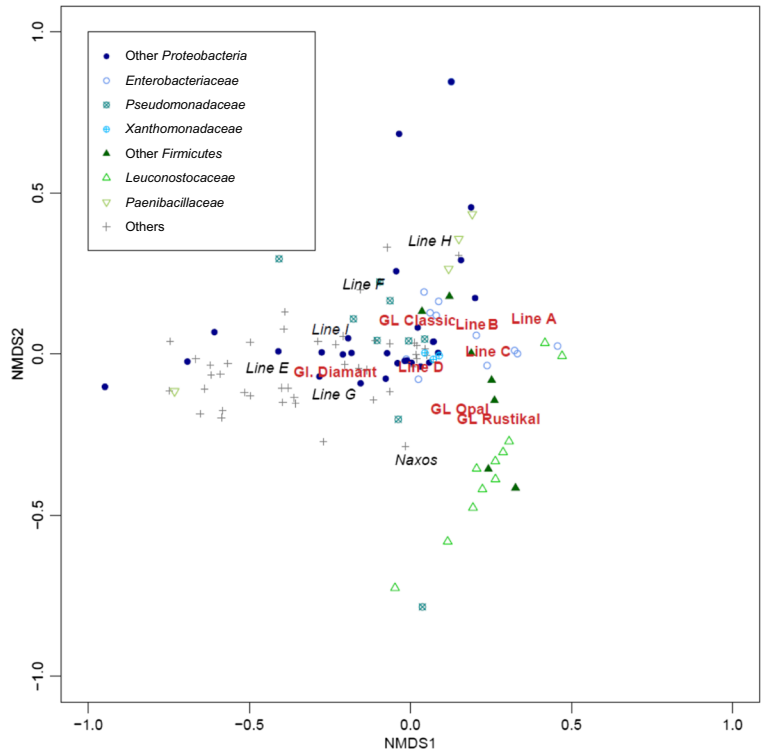


Fig. 5 Non-metric multidimensional scaling of 121 significantly different bacterial OTUs in seed microbiomes of 14 *C. pepo* genotypes. Genotypes bred in Austria are written in bold, genotypes bred in the Netherlands (Naxos), Germany (Line E), Slovenia (Line F and G) or China (Line H and I) in italics. The stress (i.e. the discrepancy between 2D configuration and predicted values from regression) was 0.159

were assigned to the family of *Leuconostocaceae* and four to *Paenibacillaceae*. A large proportion of *Leuconostocaceae* is located at similar coordinates as 'GL Rustikal' and 'GL Opal' within the NMDS plot. A weak clustering is distinguishable for the Lines A, B, C, D, 'Gleisdorfer Diamant', 'GL Classic', 'GL Opal' and 'GL Rustikal' with the geographic origin in Austria. The three cultivars with a highly diverse seed microbiome, 'Gleisdorfer Diamant', Line E and Line G, together with Line I are located at similar coordinates as the group of 'Other' bacterial taxa with significantly different abundances. According to the Shannon diversity index shown in Table 2, Line I is the fourth diverse *C. pepo* cultivar.

Specific analyses of seed microbiomes of agronomically important cultivars and of the 'GL Rustikal' pedigree

When comparing the three agronomically most important cultivars 'GL Classic', 'GL Rustikal' and 'GL Opal' with the most important component in the hybrid seed production, 'Gleisdorfer Diamant', 14% of the OTUs comprised the core microbiome (Fig. 6a). The 'Gleisdorfer Diamant' seeds were colonized with 90 unique OTUs compared to the other three genotypes investigated. Concerning the *Enterobacteriaceae* (Fig. 6b), 13 of the observed OTUs were shared within all genotypes investigated, 13 OTUs were unique to 'GL Opal', three to 'GL Rustikal' and five to 'GL Classic'. 'Gleisdorfer Diamant' seeds did not harbor unique *Enterobacteriaceae* OTUs. Three *Erwinia* OTUs were common among all four genotypes, one and three were unique in 'GL Opal' and 'GL Classic' and none were exclusively observed in 'GL Rustikal' and 'Gleisdorfer Diamant'.

To visualize the relationships of the seed associated bacterial taxa among the different genotypes a taxonomic interaction network (illustrated in Fig. 7a) of the three-way cross hybrid 'GL Rustikal' and its pedigree components (relationships shown in Fig. 7b) was created. The seed microbiome of the highly diverse 'Gleisdorfer Diamant' was comprised of 117 taxa of which 65 were unique, whereas the seed microbiomes of the other genotypes showed no (Line A, Line D, 'GL Rustikal') or just one unique taxa (Line B). The family of *Enterobacteriaceae* dominated the seed associated communities of the 'GL Rustikal' pedigree. The analysis of the OTU distribution within the 'GL Rustikal' pedigree components (Fig. 7c) revealed that 'Gleisdorfer Diamant' and Line D as parental components shared 22% and 20% of OTUs with 'GL Rustikal', whereas the genetically more distant components 'Gleisdorfer Diamant' and Line D shared 29%. The core microbiome of all five genotypes investigated was comprised of 14% of the OTUs. 14 (30%) of the observed *Enterobacteriaceae* OTUs, including three *Erwinia* OTUs, were shared within all five genotypes, which could indicate their having an essential function or that the inheritance of the microbiome is focused on certain taxa. There may be a connection of the higher bacterial diversity in 'Gleisdorfer Diamant' with the displacement and competition of *Enterobacteriaceae* in its seeds.

A Blast analysis against the NCBI nucleotide database for *Enterobacteriaceae* OTUs with an observation count of more than 10 sequences per OTU of the 'GL Rustikal' pedigree component's seed and rhizosphere samples revealed that the 16S rRNA gene sequences of most of the 54 *Enterobacteriaceae* OTUs had the highest similarity to *Klebsiella* sp. Other OTUs were assigned to *Pantoea* sp., *Salmonella* sp., *Enterobacter* sp., *Trabulsiella* sp., *Yersinia* sp., *Erwinia* sp., *Kluyvera* sp. and *Cedecea* sp. *Pectobacterium carotovorum* was part of the seed microbiome of all genotypes, except 'GL Rustikal', but was not detected in rhizosphere and bulk soil.

Discussion

Pumpkin seeds and those from related breeding lines are associated with a unique and genotype-specific microbiome. In comparison to the rhizosphere and bulk soil, microbial seed communities were characterized by a lower bacterial diversity, dominantly comprising members of *Enterobacteriaceae* including potential pathogens (*Erwinia, Pectobacterium),* but also beneficial bacteria like *Lysobacter*, *Paenibacillus,* and *Lactococcus*. In general, the data confirmed our hypothesis of a genotype-specific microbiome consisting of mainly plant-beneficial traits but several interesting and different findings will be additionally discussed.

The rhizosphere as crucial soil-plant interface was described for the first time by Lorenz Hiltner in 1904. The rhizosphere effect triggered by root exudates is also well studied (Rovira 1956) and the phenomenon that rhizosphere communities harbor less diversity than the surrounding soil was confirmed by deep sequencing

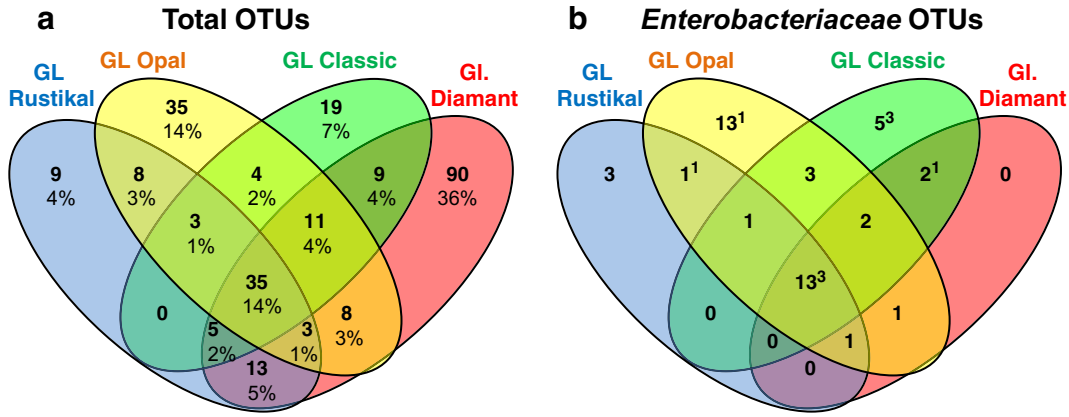


Fig. 6 OTU distribution within the seed microbiomes of **(a)** total OTUs and **(b)** *Enterobacteriaceae* OTUs of the three-way cross hybrids 'GL Rustikal' and 'GL Opal', the single cross hybrid 'Gleisdorfer Diamant' and the open-pollinated cultivar 'GL Classic'. OTU numbers of the genus *Erwinia* are in superscript. Fraction of samples that OTU was observed in to be considered as 'core': 100%

technologies (Lundberg et al. 2012; Bulgarelli et al. 2012). In our study, we showed that several taxa such as *Bacilli, Actinobacteria, Saprospirae*, *Alpha-, Beta-* and members of the *Gammaproteobacteria* were enriched in the rhizosphere microbiome, while *Thaumarchaeota, Acidobacteria*, and *Chloroflexi* occurred in higher proportions in the soil. These results fit to the conclusion that *Gammaproteobacteria,*

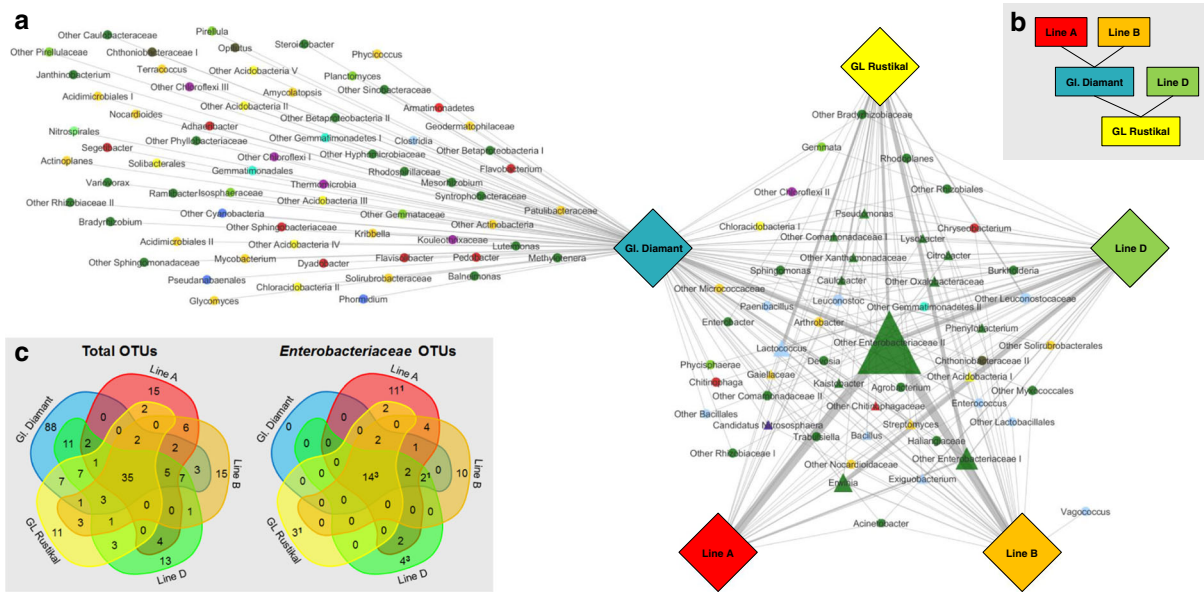


Fig. 7 **a** Taxonomic interaction network (created with the open source bioinformatics software Cytoscape (Shannon et al. 2003)) at genus level; seed associated bacterial communities of the 'GL Rustikal' pedigree and **b** the pedigree component relationships. The outer squares in the network illustrate the *C. pepo* genotypes. Seed associated bacterial taxa with a relative abundance of at least 0.1% are connected with the corresponding genotype by a grey line. The line width correlates with the relative abundance of each taxon connected with the respective genotype. The size of the shape next to the bacterial taxa corresponds to the mean of the relative abundance of all 118 bacterial taxa analyzed in this network. The 20 taxa belonging to the core microbiome are shown as triangles. Taxa that are shared by four, three or two genotypes, or that are unique in a genotype are illustrated as circles. Taxa of the same phylum are depicted in the same shape color. **c** Distribution of total OTUs within the seed microbiomes of the three-way cross hybrid 'GL Rustikal' and its pedigree components and of *Enterobacteriaceae* OTUs (fraction of samples that OTU was observed in to be considered 'core': 100%) The proportions are not drawn to scale

Betaproteobacteria and *Firmicutes* contribute to disease suppressive microbiome and that plants selectively select beneficial microorganisms (Berendsen et al. 2012). In addition to the rhizosphere effect, we can define a spermosphere, or better a seed effect, because the spermosphere only comprises the microenvironment surrounding the seeds. This seed effect is also characterized by a selective enrichment of specific microorganisms of which we assume that they are useful for germination and plant growth and health.

Traditionally, seeds were considered as carrier for pathogens only. In our study, we found potential pathogens as well as potential plant-beneficial microbes. First of all, we have to consider that 16S rDNA analyses, as performed in our study, allow only a limited prediction of the functional role. However, our analyses revealed several findings, which have to be interpreted carefully. For example, *Pectobacterium carotovorum*, an important pathogen causing fruit rot on the Styrian oil pumpkin, was part of the seed microbiome in all genotypes, with the exception of 'GL Rustikal'. The genus *Erwinia* was enriched in two genotypes. Moreover, the genus *Pseudomonas* was present in the seed microbiome of all genotypes, which comprises a number of beneficial species (Avis et al. 2008), but also includes species which can cause leaf necrosis in *Cucurbita pepo*, such as *P. vridiflava* (Grube et al. 2011; Huss and Mavridis 2007). Representatives of *X. campestris* and *P. syringae*, for example, have been shown to be seed-borne pathogens in the *Cucurbitaceae* family (Zitter et al. 1996; Robinson and Decker-Walters 1997; Babadoost and Zitter 2009; Blancard et al. 1994).

Possible beneficials like *Lysobacter* and *Paenibacillus* were also part of the seed microbiomes. Strains of both genera, *Lysobacter gummosus* L101 and *Paenibacillus polymyxa* PB71, were already used for seed treatment and resulted in significant effects on harvest yields (Fürnkranz et al. 2012). The genus *Serratia* includes potential biocontrol species as well, which led to considerable increases in germination rates of chemically untreated seeds in field experiments of the same study, but was detected in extremely low abundances in 16 out of 56 seed samples and in 6 out of 56 rhizosphere samples. In contrast, no *Serratia* signature was identified in analyzed soil samples, which indicates a specific plant-associated occurrence. Based on the results of this study, *Lactococcus* species can also be suggested as biological control agents for the Styrian oil pumpkin. This genus was highly abundant in some genotypes and Shrestha et al. (2014) reported good effects of lactic acid bacteria against *Pectobacterium carotovorum* as well as *Xanthomonas campestris*.

Due to, compared to other crops, a relatively short breeding history, the genetic differences between the *Cucurbita pepo* L. subsp. *pepo* var. *styriaca* genotypes are small, as this thin-coated seed segregant first appeared in the late nineteenth century (Teppner 2004). The commercial breeding program for the Styrian oil pumpkin in Austria at Saatzucht Gleisdorf GmbH started in 1960 and was intensified 20 years ago. Thus, it is remarkable that cultivar dependent differences in the seed microbiomes were found within this narrow gene pool. Our results contrast with the findings of Klaedtke et al. (2016), which revealed that the microbial assemblages of bean seeds were shaped by the seed production site rather than by the genotype. Within the components of the 'GL Rustikal' pedigree, the seeds of 'Gleisdorfer Diamant' were colonized by a significantly more diverse microbiome than the other genotypes, whereas for example 'GL Rustikal' seeds were characterized by a significantly greater level of colonization of *Leuconostocaceae* than other genotypes. The seed core microbiome of the cultivars analyzed in this study is dominated by high abundances of seven *Enterobacteriaceae*, one *Pseudomonadaceae*, one *Lactococcus* and one *Exiguobacterium* OTU.

The results of the seed analyses are of particular interest for the seed production industry, as the Styrian oil pumpkin is highly susceptible to various fungal and bacterial pathogens during germination making chemical or complex seed treatments inevitable. It remains to be investigated to which extent naturally occurring seed-borne bacteria influence germination and plant development. The dominance of the seed-associated microbiomes by *Proteobacteria*, and in particular *Enterobacteriaceae*, may contribute to disease susceptibility as the microbial richness and evenness of the microbial taxa are important for the maintenance of plant health (Bakker et al. 2012). The interactions of seed-borne microorganisms with the indigenous soil populations may influence the expression of biological control traits or the subsequent colonization of the rhizosphere (Nelson 2004). Therefore, the results of our study could direct the design of tailored biological seed treatments or influence seed disinfection strategies that might replace fungicide treatments in future. A possible implication for breeding programs could be the selection of genotypes enriching less enterobacteriaceal pathogens and/or expressing a higher microbial diversity in their seeds. The hologenome theory of evolution postulates that the host and its associated beneficial microbiome (holobiont) co-

evolve as one unit to provide benefits to one another including defense mechanisms (Zilber-Rosenberg and Rosenberg 2008). When postulating that breeding plants is some form of directed evolution, it may be assumed that breeding under conventional conditions (for example with the use of chemical strippers and fungicides) leads to a loss of natural defense mechanisms originally provided by the holobiont system. The results of studies like this could contribute to a paradigm shift towards ecological breeding programs.

Acknowledgments Open access funding provided by Graz University of Technology. The authors gratefully acknowledge financial support from funding of the Austrian Research Promotion Agency FFG (project no. 836466). We thank Saatzucht Gleisdorf GmbH and Alwera AG for sharing their expertise, providing seeds and the field site, as well as Barbara Fetz, Monika Schneider-Trampitsch and Alexander Fuchs for their help with sampling and Timothy Mark for English revision.

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