Drops join to make a stream: high-throughput nanoscale cultivation to grasp the lettuce root microbiome

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Running title: High-throughput root microbiome cultivation

Originality-Significance Statement

Uncontrollable environmental elements prohibit optimal food and feed production in agriculture. A plant's microbiome delivers solutions to these stresses in the form of Plant Growth-Promoting Rhizobacteria (PGPR). As most techniques for the isolation of PGPR are time- and resource consuming, we tested a new high-throughput and semi-automated bacterial isolation platform and found that the variety of bacteria isolated was complementary to more traditional techniques, thereby expanding the available cultivation methods for the study of plant-associated microbiomes.

Abstract

Root endospheres house complex and diverse bacterial communities, of which many strains have not been cultivated yet by means of the currently available isolation techniques. The Prospector® (General Automation Lab Technologies, San Carlos, CA, USA), an automated and high-throughput bacterial cultivation system was applied to analyze the root endomicrobiome of lettuce (*Lactuca sativa* L.). By using deep sequencing, we compared the results obtained with the Prospector and the traditional solid medium culturing and dilution to extinction methods. We found that the species richness did not differ and that the amount of previously uncultured bacteria did not increase, but that the bacterial diversity isolated by the three methods varied. In addition, the tryptic soy broth [TSB], and King's B [KB]) media provided a lower, but different, diversity of bacteria than that of the Reasoner's 2A [R2A] medium when used within the Prospector system and the number of unique bacterial strains did not weigh up against those isolated with the R2A medium. Thus, to cultivate an as broad variety of bacteria as possible, divergent isolation techniques should be used in parallel. Thanks to its speed and limited manual requirements, the Prospector is a valuable system to enlarge root microbiome culture collections.

Introduction

The emergence and implementation of culture-independent identification and classification methods has revolutionized our current view on the microbial tree of life (Hug *et al*., 2016; Cross *et al*. 2019). Based on the analysis of conserved marked genes (16S ribosomal (r)RNA) and metagenomics-derived data (full genomes) from divergent niches, the numbers of microbial species have been prognosticated to approach the Giga range, spanning over 60 phyla (Pedrós-Alió *et al*., 2006; Rinke *et al*., 2013). Nevertheless, only the four phyla of Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes represent approximately 88% of all microorganisms isolated to date (Hugenholtz and Kyrpides, 2009; Rinke *et al*., 2013). This inability to culture the lion's share of the bacterial diversity present on planet Earth has multiple origins, such as slow growth, specific interspecies interactions (competition, inhibition, or growth promotion) or dormancy (Adnani *et al*., 2015; Røder *et al*., 2015; Overman *et al*., 2017; Pulschen *et al*., 2017; Cross *et al*., 2019; Dong *et al*., 2020). For the alleviation of this so-called 'great plate count anomaly', various high-throughput bacterial isolation and cultivation methods have been developed with the aim to amend the traditional techniques such as plate streaking, solid medium culturing (SMC), and dilution to extinction (DTE). For instance, for high-throughput culturing and screening purposes, microfluidics are used in which single bacterial cells are encapsulated in microdroplets (Abalde-Cela *et al*., 2015; Terekhov *et al*., 2017) or the ichip (isolation chip) or similar devices are utilized, in which bacterial cells are incubated in their natural environment by placing them in diffusion chambers that are returned to nature (Berdy *et al*., 2017). Moreover, the use of different culturing media and growth conditions in combination with high-throughput identification, the so-called culturomics approach, recently gained attention (Lagier *et al*., 2016). One disadvantage most classical and modern isolation techniques have in common is that they are time consuming. As a solution to the tedious nature of traditional cultivation and the growing interest in the culturomics approach, the General Automation Lab Technologies (GALT) Prospector® system was developed, a semi-automated platform for high-throughput isolation and cultivation. Samples are loaded with a growth indicator dye on a highly dense array of 6,000 nanoscale cultivation chambers, designed for cultivation of individual microbes. The Prospector's integrated optics allow the tracking of growth on the Prospector arrays and the robotics system automatically transfers the selected microcolonies from the arrays to a standard microplate for downstream cultivation. Thus far, the Prospector has been evaluated for the isolation and cultivation of gut and soil-associated microbes, but not for plant-associated microbiomes.

High-throughput and efficient isolation and cultivation of bacteria is of great importance in multiple fields, including agriculture. A fraction of the bacteria living in and around plant roots are known as Plant Growth-Promoting Rhizobacteria (PGPR), recognized for their ability to confer, among others, abiotic stress relieve to plants (Ilangumaran *et al*., 2017; Bharti *et al*., 2016; Enebe and Babalola, 2018; Goswani and Deka, 2020). As we are interested in understanding how the root microbiome contributes to cold resistance in crops (Beirinckx *et al*., 2020), we focused on the isolation of the root microbiome of lettuce (*Lactuca sativa*) plants grown at low temperature and under control conditions. Even though global warming is a fact, the increasing temperatures distort air currents around the planet's North pole, allowing cold air to escape to the continents below. These drops in temperature are financially unfavorable for farmers who cultivate tender crops under greenhouse conditions, because they require constant heating during the colder months to support a year round supply. A sustainable approach to alleviate high heating costs might be found in the use of PGPR that promote plant growth under reduced temperature conditions. For instance, *Burkholderia phytofyrmans* PsJN has been shown to acclimate both grapevine (*Vitis vinifera*) and *Arabidopsis thaliana* to the cold, whereas other bacterial strains have been found to promote the growth of maize (*Zea mays*) and tomato (*Solanuim lycopersicum*) seedlings under low temperature conditions (Fernandez *et al*., 2012; Wang *et al*., 2016; Beirinckx *et al*., 2020)

Here, we compared the Prospector and classical isolation techniques to evaluate the Prospector's contribution to the isolation of the lettuce root endosphere-associated microbiome for the assembly of a diverse bacterial collection. This collection could then be used to test the bacteria for their growth-promoting properties on lettuce under low temperature conditions (see Supporting Information for details on Experimental Procedures).

Results and discussion

To evaluate whether the Prospector system was complementary to SMC and DTE, we set up a microbiome-based comparison of the three different cultivation methods with the uncultured root endosphere communities. The roots of two lettuce cultivars, grown under cold and control temperature conditions, were used as starting material. Firstly, the Prospector, SMC and DTE were combined with the R2A medium in order to compare the three techniques. Secondly, within the Prospector system, three different cultivation media (R2A, KB and TSB) were assessed for their influence on the diversity of the isolated bacteria. In both experimental set-ups, 400 colonies were randomly picked under each

condition and all samples were subjected to metabarcoding of the V4 region of the 16S rRNA gene (Fig. 1).

The obtained libraries yielded 647 ASVs belonging to 180 different known bacterial genera and the overall community structure was assessed by alpha and beta diversity analysis. The alpha (within sample) diversity was estimated by calculating the Shannon diversity index on rarefied data, taking into account richness and evenness of groups. As expected, the uncultured samples had an alpha diversity exceeding that of all cultured samples (paired Wilcoxon rank test, $P<0.001$), followed by generally equivalent alpha diversities for bacteria cultured with DTE and SMC ($P>0.05$) and a slightly lower diversity for the Prospector system when combined with the R2A medium ($P<0.01$). The lowest diversity was observed for samples cultured in TSB and KB, albeit not statistically different from each other ($P>0.05$) but with a significantly lower alpha diversity than that of DTE, SMC and the Prospector with the R2A medium ($P<0.001$) (Fig. 2A). The beta (between sample) diversity was obtained by means of a Principal Coordinates Analysis (PCoA) on a Bray-Curtis dissimilarity matrix. Furthermore, visual inspection of the PcoA plot revealed that the beta diversity from the uncultured samples distinctively separated from the cultured ones, with subtle shifts from the bacteria cultured with DTE to those with SMC and finally with the Prospector system (Fig. 2B). Based on statistical analyses, the method used had a more significant effect on the diversity between samples ($R^2 = 0.29350$ and $P< 0.001$) than that of the growth medium used ($R^2 = 0.09356$ and $P< 0.001$). Thus, both the alpha and beta diversities are predominantly influenced by the isolation/culturing method used, as shown in literature (Tanaka *et al*., 2014; Medina *et al*., 2017; Stevens *et al*., 2021). An additional factor influencing the beta diversity is the growth temperature (Fig. 2C) (Beirinckx *et al*., 2020). Temperature had a prominent effect on the uncultured root endosphere samples, in contrast to the cultured sample, in which its impact was more subtle or even absent. However, this effect will not be taken into account, because our research is focused on differences between isolation techniques, independently of temperature.

The SILVA release 138 database was used for taxonomy assignment of the identified ASVs, allowing a more in-depth investigation into the bacterial community composition of the uncultured samples and samples derived from the different culturing methods. Consistently with the previously described lettuce endomicrobiomes (Cardinale *et al*., 2015; Zhang *et al*., 2018), more than 65% of the ASVs in the uncultured samples were assigned within the phyla Proteobacteria (43.5 %, subdivided in 31.5% Gammaproteobacteria and 12.5% Alphaproteobacteria), Actinobacteria (5%) and Bacteroidota (17.7%). The remaining ASVs (34,3%) were classified into another 13 phyla (Verrucomicrobiota,

Myxococcota, Chloroflexi, Planctomycetota, Spirochaetota, Acidobacteriota, Bdellovibrionota, Patescibacteria, Armatimonadota, Latescibaterota, Cyanobacteria, Elusimicrobiota and candidate phylum FCPU426) (Fig. 3A). In contrast, all the diversity found in the cultured samples was assigned exclusively to the four well-known cultivable phyla Proteobacteria, Bacteroidota, Actinobacteriota and Firmicutes (Khan Chowdhury *et al*., 2017; Tanaka *et al*., 2017; Chaudhary *et al*., 2019; Acuña *et al*., 2020; Stevens *et al*., 202). When relative abundances of the Prospector-guided isolations in the R2A medium were compared to those by DTE and SMC, the Prospector technology isolated primarily a variety of Proteobacteria (97%), followed by small fractions of Bacteroidota (2.7%) and Actinobacteria (0.3%), whereas both the DTE and SMC methods isolated a larger fraction of Bacteroidota (35% and 33%, respectively) and low amounts of Actinobacteria (1% and 2%, respectively). In contrast to the Prospector technology, both methods isolated bacteria belonging to the Firmicutes phylum with much larger proportions, being 10% for the SMC and 1% for the DTE method compared to <1% with the Prospector.. It is noteworthy that all three cultivation methods isolated almost exactly the same proportions of Gammaproteobacteria and Alphaproteobacteria (on average 68% and 31%, respectively). Comparison of the different media used within the Prospector technology clearly indicated that the KB and R2A media selected both Alphaproteobacteria and Gammaproteobacteria (43% and 57% for KB and 33% and 67% for R2A, respectively), whereas the TSB medium favored growth of almost exclusively Gammaproteobacteria (93%) (Fig. 3B). All media isolated Bacteroidota with the largest fraction for TSB (5%) and the smallest for KB (1%). Finally, the R2A and TSB media isolated a minor fraction of Actinobacteriota and TSB was the only medium that isolated bacteria from the phylum Firmicutes when the Prospector technology was used.

At the genus level, we found that an average of 38.8% of the ASVs in the uncultured samples could not be classified, in accordance with the generally described plant-associated microbiomes (Fernández-González *et al*., 2019; Lasa *et al*., 2019; Toju *et al*., 2019) (Fig. 4A), whereas in the cultured samples, 9.5% of the bacteria grown with SMC and less than 8% under all other conditions could not be classified, suggesting that the traditional SMC method still yields potentially most novel species in this set-up. When the relative abundances of the top 10 most abundant genera in the three different cultivation methods are considered, the classical methods clearly strongly favored growth of *Flavobacterium* (34% for DTE and 30% for SMC *versus* 2% for the Prospector method), whereas the opposite is true for *Pseudoxanthomonas* (23% for the Prospector compared to 3% for both the DTE and SMC methods). These observations suggest that *Flavobacterium* do not develop under the contained Prospector conditions, whereas the *Pseudoxanthomonas* species seem to thrive in them. The

Prospector system also favored growth of *Rhizobium* (17% compared to 6% and 10% for DTE and SMC, respectively), *Stenotrophomonas* (16% *versus* 1% and 12% for DTE and SMC, respectively) and *Rhizorhapis* (6% *versus* 3% and 0.5% for DTE and SMC, respectively). Hence, the Prospector system allows the isolation of a complementary group of strains to those isolated with the SMC and DTE methods. This complementarity in culturability between different groups of bacteria based on the cultivation method had previously been observed, reinforcing the necessity of combining divergent isolation approaches to retrieve the highest possible diversity of microbes (Stefani *et al*., 2015; Bilen *et al*., 2018; Chaudhary *et al*., 2019; Bonnet *et al*., 2020).

Regarding the three different culturing media used within the Prospector system, all isolated nearly equal amounts of *Stenothropomonas* and *Pseudomonas* (on average 20%), two genera known for their cultivability on a wide variety of growth media (LaMontagne *et al*., 2021) (Fig. 4B). The KB medium favored growth of *Rhizobium* (38% *versus* 15% and 4% for R2A and TSB, respectively) whereas the TSB medium supported cultivation of *Klebsiella* species (38% *versus* 4% and 0.5% for KB and R2A, respectively). *Rhizorhapis* grew primarily on the R2A medium (7%).

Next, weighted Venn diagrams, based on the ASV ID, were constructed to demonstrate the percentage of common and unique ASVs for each of the three cultivation methods and the different culturing media used within the Prospector system (Fig. 5). As more than half (56.8%) of all ASVs isolated were unique for the different methods used, the importance of using different approaches for bacterial cultivation is emphasized. The Prospector yielded the highest number of ASVs unique to the method used (49) and shared relatively few ASVs with both the SMC and DTE methods (9 and 12, respectively), whereas the latter shared the highest overlap of ASVs across the methods (31). From the isolates unique to the SMC method, eight ASVs were not classified and thus can be regarded as possibly novel, compared to seven and four for the DTE and Prospector methods, respectively. Even though the number of previously uncultured bacteria isolated by the Prospector system was not higher than that by the traditional isolation methods, it remains complementary by culturing many bacteria not isolated with the traditional techniques (Fig. 5A). Concerning the different culture media used within the Prospector system, 58.9% of all ASVs isolated was unique to the medium used, 18,7% was shared between two media and 22.3% was common for the three different media (Fig. 5B). Together with the abovementioned findings, these results highlight the importance of using different culturing methods and media to isolate an as wide variety of bacteria from a certain niche as possible.

Fig. 6 depicts the phylogenetic relationship of all ASVs from the uncultured samples belonging to the four major cultivable phyla and presents the percentage of uncultivated and cultivated ASVs for

the three different cultivation methods used in combination with the R2A medium, within each phylum. For bacteria belonging to the Actinobacteriota, Bacteroidota and Alphaproteobacteria, on average 50% of the ASV detected in the uncultured samples remained uncultured, confirming the endorsed finding that R2A is not the ideal isolation medium for some members of these phyla (Undabarrena *et al*., 2016). Concerning Gammaproteobacteria, 38% of the ASVs detected in the uncultured samples were not isolated by one of the three cultivation methods, whereas this was only 26% for members of the Firmicutes phylum. The high percentage of cultivability for this phylum could be assigned to their spore-forming abilities, enabling them to respond quickly and efficiently to nutrient availability and thus outgrow the competition, possibly the reason why 41% of the isolates grew on solid medium (Parkes and Sass, 2009). The numbers of SMC specific ASVs remained low for all other phyla, except for members of the Actinobacteriota, with an average of 3.6% and 21%, respectively. Actinobacteriota are known for their biofilm forming capabilities, both in solid and liquid cultures. Absence of active shaking may have caused sedimentation and attachment of colonies to the bottom of both the DTE and Prospector wells, preventing adequate transfer of colonies, explaining the higher percentage of SMC specific ASVs. The DTE unique isolates, with an average of 5.8%, remained constant over all phyla. In contrast to both classical isolation methods, the Prospector isolated the highest number of unprecedented ASVs (on average 11.75%) for three out of the four phyla, with the exception of 4% for members of the Firmicutes. The spore-forming Firmicutes strains might sense the low nutrient availability in the extremely small volumes of the Prospector array, inhibiting them from breaking dormancy. By contrast, members of the Proteobacteria appear to grow best in small volumes.

Conclusions

Bacterial collections are indispensable means to evaluate single strains for their growth-promoting properties. Here, we evaluated the high-throughput and automated bacterial isolation and cultivation tool Prospector (GALT) in comparison with the more traditional SMC and DTE methods and found that it did not isolate a more diverse collection of bacteria than the traditional methods, but all did have a different strain diversity. In combination with the speed and limited resource requirements by which high numbers of bacteria can be isolated, the Prospector is a valuable system to enlarge root microbiome culture collections. Summarized, these results emphasize the importance of implementing divergent isolation techniques in parallel to cultivate an as broad variety of bacteria as possible.

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345

346 **Supporting Information**

347

348 Additional Supporting Information may be found in the online version of this article at the
349 publisher's web-site:
350 **Appendix 1**. Experimental procedures.
351

Figure Legends

Fig. 1 Experimental set-up used to evaluate the performance of the Prospector system as a novel tool to isolate the lettuce root endosphere.

Fig. 2 Alpha- and beta diversity patterns of cultured and uncultured samples of the lettuce root endosphere microbiome. Data of the 16S rRNA V4 region were analyzed. (**A**) Alpha diversity assessed with Shannon's diversity. Boxplots show richness, Shannon diversity index, and evenness with median and upper and lower quartiles; dots represent individual samples. (**B, C**) Beta diversity assessed by a PCoA on a Bray-Curtis dissimilarity matrix. Loops group samples based on the method used to assess their bacterial community.

Fig. 3: Bacterial taxonomic community structure of (**A**) cultured and uncultured samples and (**B**) different growth media used within the Prospector system at the phylum level. Relative abundances of all detected phyla are depicted. The surface area above and below the dotted lines indicates the proportion of the Gamma- and Alphaproteobacteria within the phylum Proteobacteria, respectively (n = 24 for each condition).

Fig. 4 Bacterial taxonomic community structure of (**A**) cultured and uncultured samples and (**B**) different growth media used within the Prospector system at the genus level. Relative abundances of the top nine most abundant genera are shown (n= 24 for each condition).

Fig. 5 Weighted Venn diagrams depicting shared and unique ASVs isolated from the lettuce root endosphere with (**A**) three different bacterial cultivation methods and (**B**) three different culture media used within the Prospector system.

Fig. 6 Phylogenetic tree (maximum likelihood) based on the V4 (515-806) region of the 16S rRNA gene showing the taxonomic affiliation all ASVs in the uncultured root endosphere samples from the phyla Actinobacteriota (red), Bacteroidota (green), Alphaproteobacteria (light blue), Firmicutes (yellow), and Gammaproteobacteria (dark blue). For each phylum the percentage of uncultured and cultured ASVs is indicated with a corresponding color code (1,000 bootstrap replicates were used, the scale bar represents 0.1 substitutions per nucleotide position).

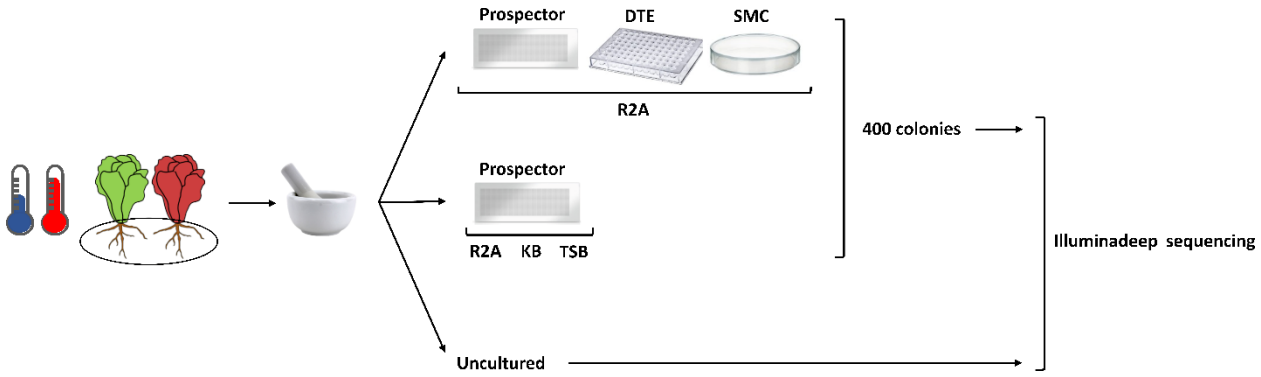
Prospector
DTE
SMC
R2A
Prospector
R2A
KB
TSB
400 colonies
Illuminadeep sequencing
Uncultured

Fig. 1.

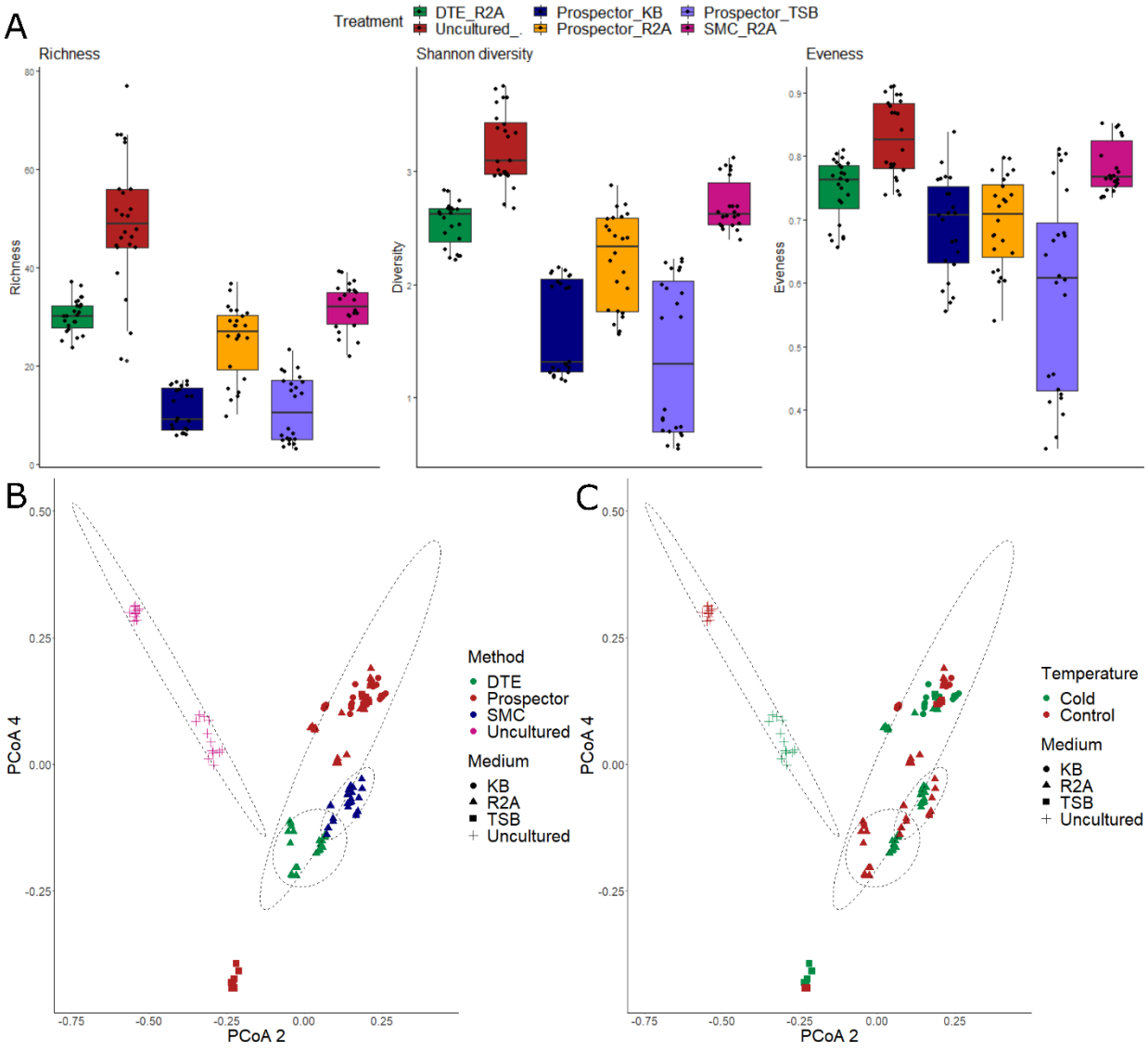
A
Treatment
DTE_R2A
Uncultured_.
Prospector_KB
Prospector_R2A
Prospector_TSB
SMC_R2A
Richness
Shannon diversity
Eveness
Richness
Diversity
Eveness
B
C
PCoA 4
PCoA 2
Method
DTE
Prospector
SMC
Uncultured
Medium
KB
R2A
TSB
Uncultured
Temperature
Cold
Control
Medium
KB
R2A
TSB
Uncultured

Fig. 2.

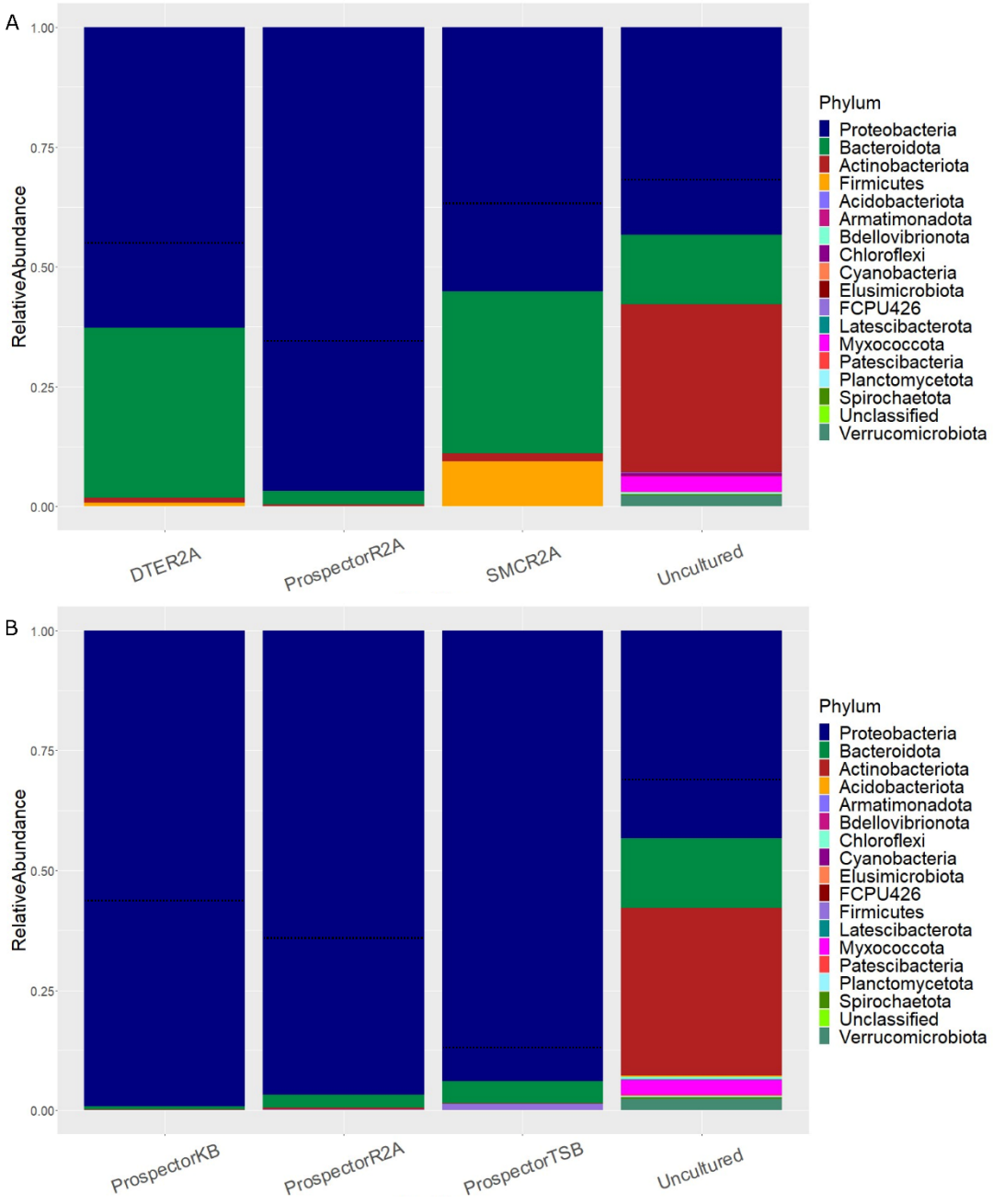
A
1.00
0.75
0.50
0.25
0.00
RelativeAbundance
DTER2A
ProspectorR2A
SMCR2A
Uncultured
Phylum
Proteobacteria
Bacteroidota
Actinobacteriota
Firmicutes
Acidobacteriota
Armatimonadota
Bdellovibrionota
Chloroflexi
Cyanobacteria
Elusimicrobiota
FCPU426
Latescibacterota
Myxococcota
Patescibacteria
Planctomycetota
Spirochaetota
Unclassified
Verrucomicrobiota
B
1.00
0.75
0.50
0.25
0.00
RelativeAbundance
ProspectorKB
ProspectorR2A
ProspectorTSB
Uncultured
Phylum
Proteobacteria
Bacteroidota
Actinobacteriota
Acidobacteriota
Armatimonadota
Bdellovibrionota
Chloroflexi
Cyanobacteria
Elusimicrobiota
FCPU426
Firmicutes
Latescibacterota
Myxococcota
Patescibacteria
Planctomycetota
Spirochaetota
Unclassified
Verrucomicrobiota

Fig. 3.

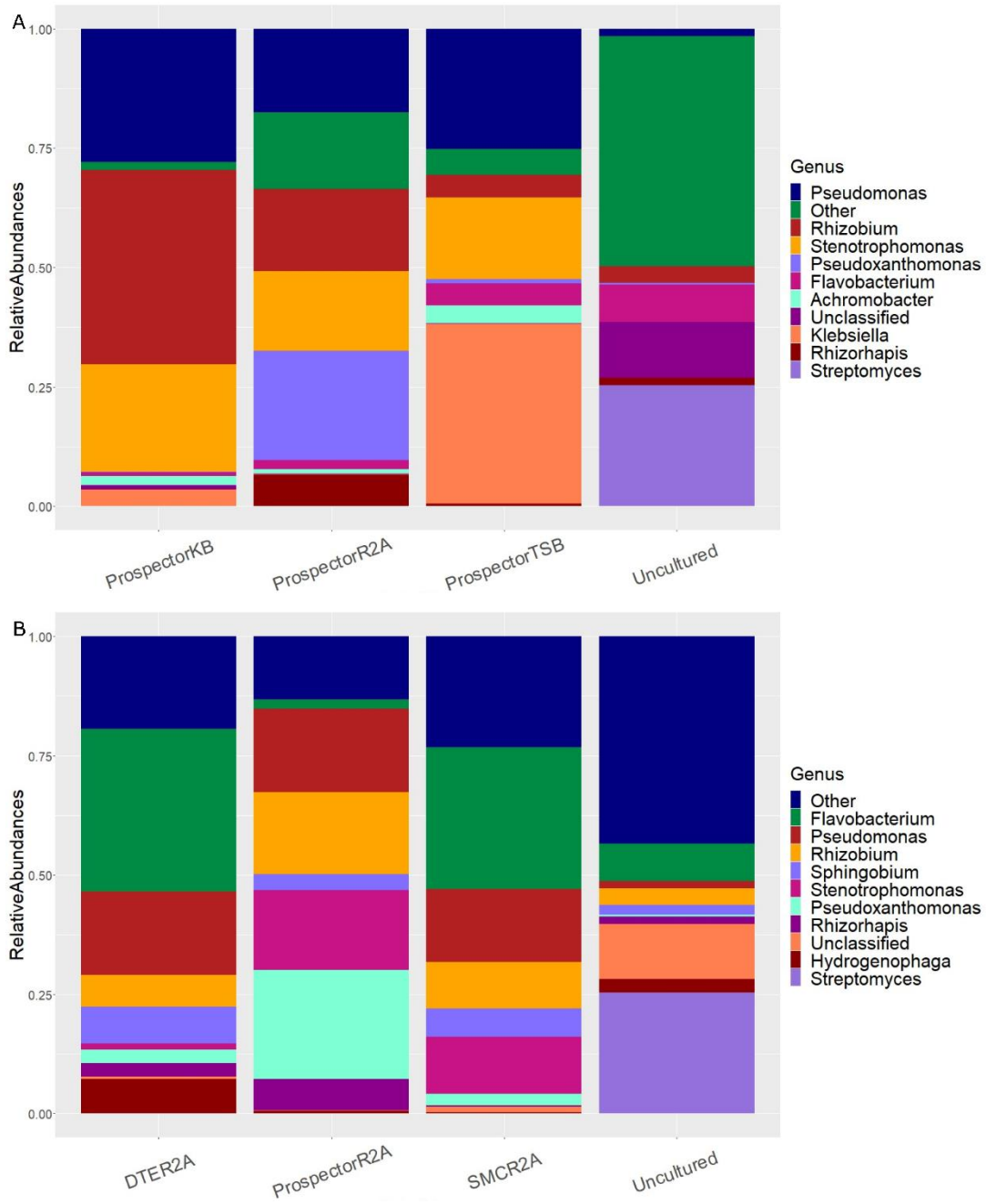
A
1.00
0.75
0.50
0.25
0.00
RelativeAbundances
ProspectorKB
ProspectorR2A
ProspectorTSB
Uncultured
Genus
Pseudomonas
Other
Rhizobium
Stenotrophomonas
Pseudoxanthomonas
Flavobacterium
Achromobacter
Unclassified
Klebsiella
Rhizorhapis
Streptomyces
B
1.00
0.75
0.50
0.25
0.00
RelativeAbundances
DTER2A
ProspectorR2A
SMCR2A
Uncultured
Genus
Other
Flavobacterium
Pseudomonas
Rhizobium
Sphingobium
Stenotrophomonas
Pseudoxanthomonas
Rhizorhapis
Unclassified
Hydrogenophaga
Streptomyces

Fig. 4.

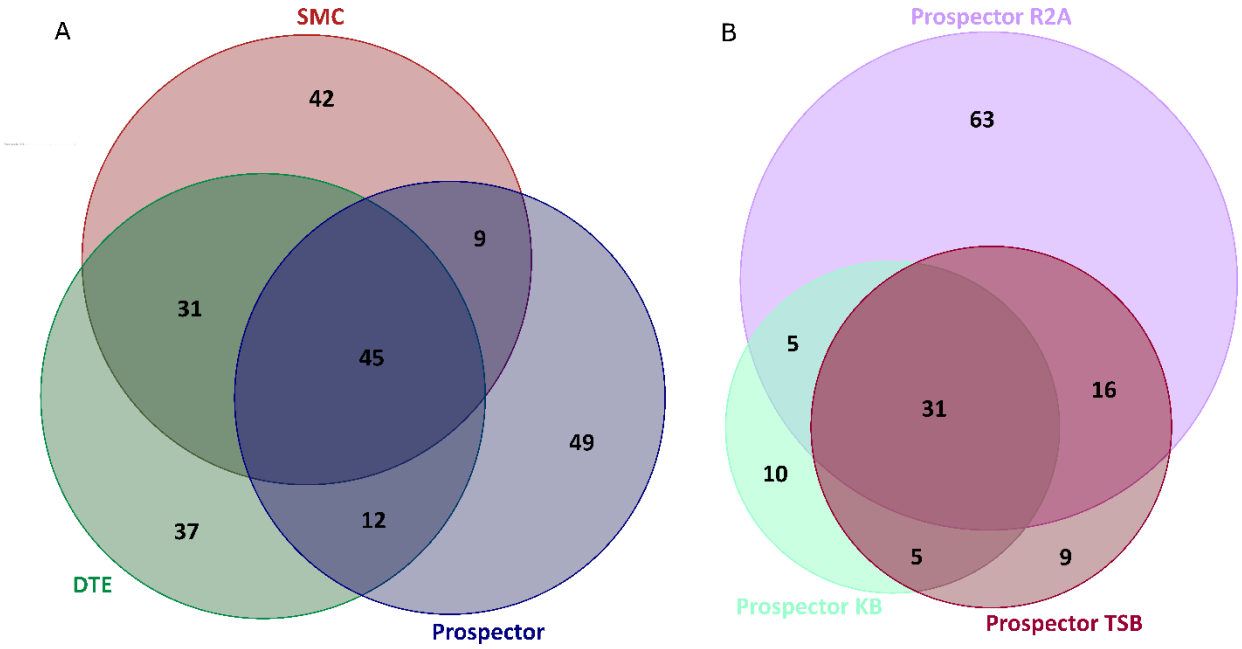
A
SMC
42
9
31
45
49
37
12
DTE
Prospector
B
Prospector R2A
63
5
31
16
10
5
9
Prospector KB
Prospector TSB

Fig. 5.

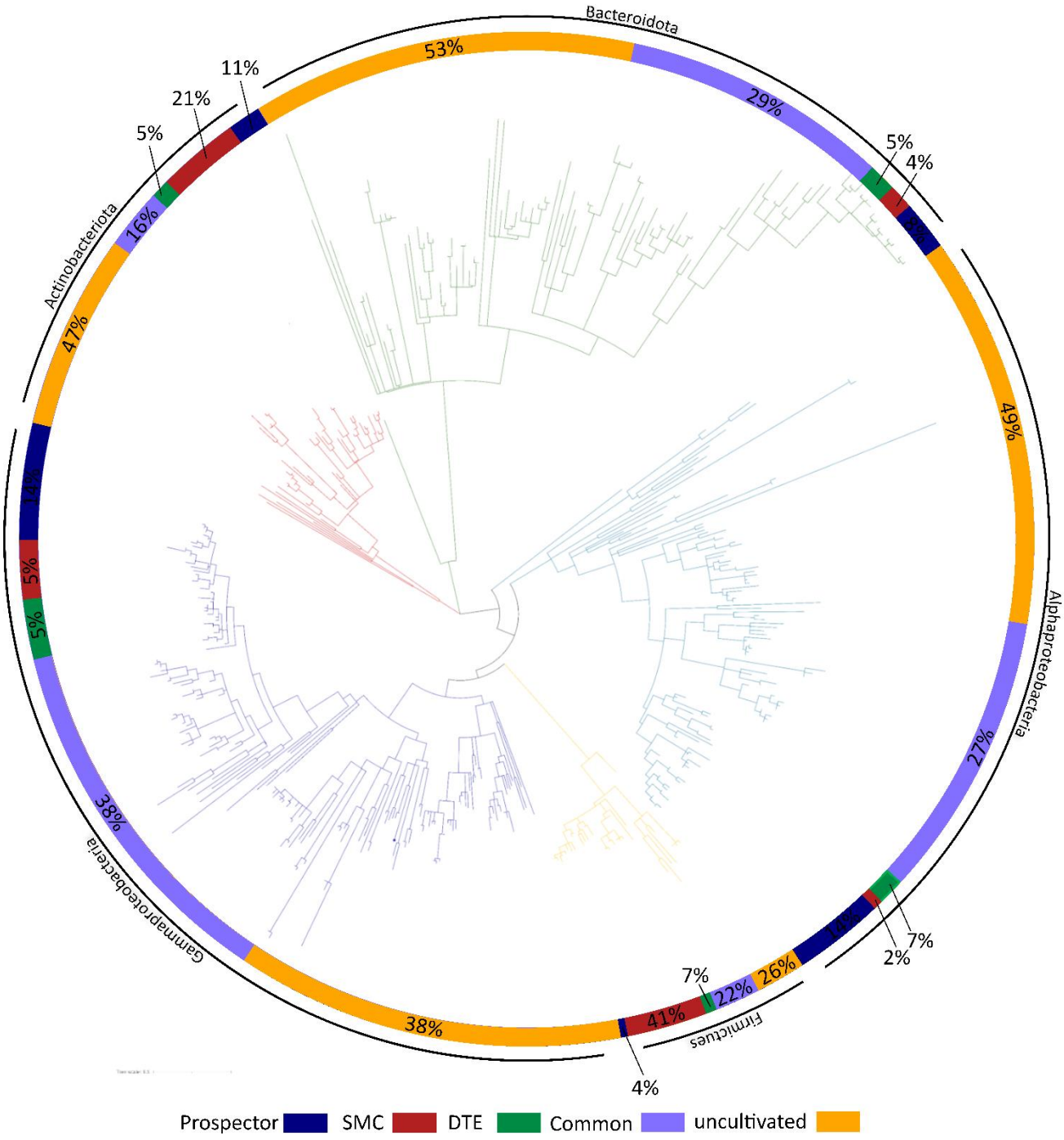
Bacteroidota
53%
29%
5%
4%
5%
Alphaproteobacteria
49%
27%
7%
2%
4%
Firmicutes
7%
22%
26%
14%
41%
4%
Gammaproteobacteria
38%
38%
14%
5%
5%
Actinobacteriota
47%
16%
5%
21%
11%
Prospector
SMC
DTE
Common
uncultivated

Fig. 6.