Do size and shape matter? Exploring the interactions and the metabolome of the soil isolate *Hylemonella gracilis*

Olaf Tyc[1,2*], Purva Kulkarni[1,3], Adam Ossowicki[1], Vittorio Tracanna[4], Marnix H. Medema[4], Peter van Baarlen[5], W.F.J. van Ijcken[6], Koen J. F. Verhoeven[1] and Paolina Garbeva[1, 7]

[1]Netherlands Institute of Ecology (NIOO-KNAW), Department of Microbial Ecology, PO BOX 50, 6700 AB Wageningen, Netherlands

[2]Goethe University, Department of Internal Medicine I, University Hospital Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany

[3]Translational Metabolic Laboratory, Radboud University Medical Center, Geert Grooteplein zuid 10, 6525 GA Nijmegen, Netherlands

[4]Wageningen University, Department of Plant Sciences, Bioinformatics Group, Droevendaalsesteeg 1, 6708PB Wageningen, Netherlands

[5]Wageningen University, Department of Animal Sciences, Host-Microbe Interactomics, Postbus 338, 6700 WD Wageningen, Netherlands

[6]Center for Biomics, Erasmus University Medical Center, Building Ee, Room Ee6.79b Wytemaweg 80, 3015 CN Rotterdam Rotterdam, The Netherlands

[7]Department of Plant and Environmental Sciences, Faculty of Natural and Life Sciences, University of Copenhagen, Denmark

Correspondence:
Goethe University, Department of Internal Medicine I, University Hospital Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany, E-Mail: olaf.tyc@kgu.de

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Abstract

Microbial community analysis of aquatic environments showed that an important component of microbial diversity consists of bacteria with cell sizes smaller than ~0.1 µm. However, so far no study investigated if such bacteria with small cell sizes exist in terrestrial environments as well.

Here, we isolated soil bacteria that passed through a 0.1 µm filter, by applying a novel isolation and culturing approach. The complete genome of one of the isolates was sequenced and the bacterium was identified as *Hylemonella gracilis*. We performed a set of interaction assays with phylogenetically distant soil bacteria with larger cell and genome size. The interaction assays revealed that *H. gracilis* grows better when interacting with other soil bacteria like *Paenibacillus* sp. AD87 and *Serratia plymuthica*. Furthermore, we observed that *H. gracilis* is able to change the behavior of interacting bacteria without direct cell-cell contact. Transcriptomics and metabolomics analysis was performed with the aim to explain the mechanisms of these interactions.

Our study indicates that soil bacteria that can pass through a 0.1 µM filter may have been overlooked in soil microbial communities and that such bacteria are able to induce transcriptional and metabolomics responses in other soil-bacteria. Furthermore, we revealed that the interaction allowed utilization of substrates that are not utilized by monocultures.

Introduction

Bacteria are ubiquitous living organisms with various cell shapes and sizes surrounding us in all environments (1, 2). Soil is the most complex habitat harboring the largest diversity and density of bacteria known to date (cell densities ranging from 10^7 to 10^{10} cells/g of soil (3-5). Soil bacteria are part of a community where they are in constant interaction with their own and other species (6-8). Bacteria produce and release a plethora of metabolites into their environment. In this way, they not only modify their niche but also affect the behavior and the secondary metabolite production of nearby bacteria (9-11). Soil bacteria are known to produce both soluble and volatile secondary metabolites with different physicochemical and biological properties (7, 12-14). In contrast to soluble compounds, volatile organic compounds (VOCs) are rather small molecules (< 300 Da) that can diffuse easily through air- and water-filled soil-pores, (15-17). These physiochemical properties make VOCs ideal metabolites for long-distance communication and interactions between soil microorganisms (18-21).

In aquatic environments, bacteria are naturally found at lower cell density of 10^3 - 10^6 cells/mL (22-24). Recent studies have shown that a significant component of microbial diversity consists of bacteria with cell sizes smaller than ~0.1 µm (25-27). However, little is known about bacteria with such small cell sizes in soil environments. One can assume that small cell size can be an advantage in challenging environments like soil. The distribution of microorganisms in soil is influenced by its water and moisture content, a low soil moisture content leads to lower connectivity between soil pores, and thus to a lower number of accessible micro-habitats.

Small bacterial cell size is often linked to a small genome size caused by genome streamlining (28). Recent metagenomics studies suggest that genome streamlining is ubiquitous in many bacteria (29, 30). In some cases, the primary metabolism of one organism can be directly built on the primary metabolism of another organism, known as syntrophic relationships (31, 32). The Black Queen Hypothesis states that genome-streamlined organisms have an evolutionary advantage because of the loss of genes whose function can be replaced by bacteria in the surrounding environment, effectively conserving energy (33). Since bacteria with smaller genomes have less adaptive capacity compared to bacteria with bigger genome sizes, many of them depend on specific environmental conditions and the presence of other specific organisms (34) to produce metabolites that support their persistence.

Here, we explore if soil bacteria that are able to pass through 0.1 µm filters are present in soil and we investigated their interaction with other soil bacteria. The major research questions were if, and how inter-specific interactions between bacteria smaller than 0.1 µM and other common

soil bacteria affected fitness, behavior, gene expression, and the production of secondary metabolites.

Materials and Methods

Isolation and identification of bacteria that pass through 0.1 µm filters

Please see **Fig. S1** and Supplementary Methods.

Identification of bacteria that passed through 0.1 µm filter

One type of bacterial colony was observed on the inoculated plates. The grown colonies were later identified as *Hylemonella gracilis* by 16S rRNA sequence analysis. Please see Supplementary Methods.

Bacteria and culture conditions

Please see Supplementary Methods and **Supplementary Table 1**.

Microscopy

Please see **Supplementary Methods.**

Bacterial interactions assays on 1/10th TSBA plates

For the interaction assay, liquid bacterial cultures were diluted to an OD_{600} of 0.005 (*Paenibacillus* and *Serratia*) or to an OD_{600} of 0.05 (*H. gracilis*). A 10 µl droplet was added in the middle of a 6 cm diameter Petri dish (monocultures) or next to each other in a distance of ~0.5 cm (pairwise interactions), for details please see Supplementary Methods.

Effects of cell-free supernatants of *Paenibacillus* and *Serratia* on the growth of *H. gracilis*

Please see Supplementary Methods.

DNA isolation and genome sequencing of *H. gracilis*

Genomic DNA of *H. gracilis* was extracted using a QIAGEN Genomic-tip 500/G DNA kit Qiagen, cat# 10262 (for details see Supplementary Methods). The genome sequencing was performed on the PacBio RS II platform (Pacific Biosciences, Menlo Park, CA, USA) using P6-C4 chemistry at the Institute for Genome Sciences (IGS), Baltimore, Maryland, USA. The sequencing resulted in a total of 70,101 reads with N50 of 17 309 nucleotides. The PacBio raw sequences were analyzed using SMRT portal V2.3.0.140936. p.4150482. Sequences were

assembled *de novo* with the RS_HGAP_assembly 3 protocol (© Copyright 2010 - 2014, Pacific Biosciences, Menlo Park, CA, USA) with default settings on an estimated genome size of 3.8 Mbp. The resulting assemblies were subjected to scaffolding using the RS_AHA_scaffolding 1 protocol. The genome assembly properties are shown in **Supplementary Table 2**. The final contigs were annotated using PROKKA V1.11 (42) and InterproScan 5.16 55.0 (43). The whole genome sequence was submitted as *Hylemonella gracilis* strain NS1 to NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under accession # CP031395.

In silico analysis of secondary metabolite gene clusters

For *in silico* analysis of secondary metabolite gene clusters, the genome sequences of *H. gracilis, Paenibacillus sp.* AD87 and *Serratia plymuthica* PRI2C were submitted to the antiSMASH web server (http://antismash.secondarymetabolites.org/) version 4.0 (44).

RNA sampling, isolation and RNA- sequencing

Please see Supplementary Methods.

Pathway annotations

Please see Supplementary Methods.

Exploration of missing genes and genome streamlining in *Hylemonella*

RAST annotations of *Serratia*, *Paenibacillus* and *H. gracilis* were used to compare the genomes and to explore the genomes for missing genes in metabolic pathways (http://rast.nmpdr.org) (52-54). RAST was used to identify missing core genes in genomes. The missing gene sequences were extracted and assigned with KEGG Orthology (50, 55). Presence/absence of genes belonging to metabolic pathways was compared across the three genomes to identify shared genes and pathways and to determine incomplete metabolic pathways in *H. gracilis*.

Catabolic profiling using BioLog EcoPlate™

Please see Supplementary Methods.

Trapping of volatile organic compounds and GC-Q-TOF analysis

Please see Supplementary Methods.

Ambient mass-spectrometry imaging analysis (LAESI-MSI)

Please see Supplementary Methods.

Analysis of Ambient mass-spectrometry imaging (LAESI-MSI) Data

Please see Supplementary Methods.

Data availability

The raw data of this article will be made available by the authors, to any qualified researcher. The whole genome sequence of *Hylemonella* strain NS1 is available at the NCBI GenBank under accession # CP031395, the raw reads of the transcriptomics data are available at the Sequence Read Archive (SRA) https://www.ncbi.nlm.nih.gov/sra under accession # SUB8619726.

Results

Isolation and identification of bacteria that pass through 0.1 µM filter

Using a novel bacterial isolation and cultivation approach, we isolated bacteria from a terrestrial soil sample able to pass through a 0.22 µm and a 0.1 µm pore-size filters. The bacteria were identified as *Hylemonella gracilis* (Gram-negative, class betaproteobacteria, order Burkholderiales). The colonies showed a round and colorless morphology when grown on $1/10^{th}$ TSBA plates (**Fig. 1a**). Microscopically the bacteria had a spiraled morphology with a length of approximately 6 - 12 µM which is typical for *Hylemonella* species (**Fig. 1b**).

Hylemonella grows better in interaction with other bacteria

H. gracilis growth was determined during the interaction with two phylogenetically distantly related soil bacteria (*Paenibacillus* sp. AD87 and *Serratia plymuthica* PRI-2C) and compared to that of the monoculture. The bacterial colony forming units of *H. gracilis* (CFU/mL) obtained on 1/10th TSBA plates from monocultures and interactions are summarized in **Fig. 2**. Cell counts of *Paenibacillus* were 7.68 x 10^7 CFU/mL in interaction with *H. gracilis* (**Fig. 2a**). During the interaction with *H. gracilis*, the growth of *Serratia* was significantly negatively affected (*P=0.037*) after five days of incubation by reaching 1.47 x 10^9 CFU/mL compared to the monocultures (**Fig. 2a**).

The bacterial colony forming units (CFU) obtained from *H. gracilis* grown in presence of cell free supernatants (CFS) of *Paenibacillus* and *Serratia* are summarized in **Fig. 2b**. *H. gracilis* growth was significantly increased (*P*=0.011) when growing in presence of cell free

supernatants of *Paenibacillus* resulting in higher cell counts compared to the monoculture by reaching 1.10 x 10^6 CFU/mL. In the presence of cell free supernatant from *Serratia, H. gracilis* reached the highest cell counts at 1.72 x 10^6 CFU/mL (P=0.000) after five days of incubation (**Fig. 2b**).

Interaction between bacterial species allows use of substrates that are not used in monoculture

The catabolic profiling using EcoPlates™ revealed that *Paenibacillus* was able to utilize 11 of the 31 carbon sources in monoculture, *Serratia* and *H. gracilis* were able to utilize 17 and 16 carbon sources respectively. Interestingly, three compounds could be utilized only during co-cultivation of *H. gracilis* with one of the other species, these compounds could not be utilized by any of the species in monoculture. Specifically, alpha- cyclodextrin was utilized only during co-cultivation of *H. gracilis* with *Paenibacillus*, while L-threonine and glycyl-L-glutamic acid were utilized only during the interaction of *Serratia* and *H. gracilis* **Fig. 3.**

Genomic features of *H. gracilis, Serratia and Paenibacillus*

Sequencing of the complete genome of *H. gracilis* resulted in a genome size of 3.82 Mbp with 3,648 coding sequences (CDS). As expected, the genome analysis revealed that the genome of *Hylemonella* is smaller and contains fewer genes when compared to both *Serratia plymuthica* PRI-2C (5.4 Mbp) and *Paenibacillus* sp. AD87 (7 Mbp). The genome features of all three bacteria are summarized in **Supplementary Table 2**.

***In silico* analysis of gene clusters encoding for secondary metabolites**

In silico analysis of *Paenibacillus* revealed a total of 10 gene clusters coding for secondary metabolites. From which two gene clusters belonged to the class of terpenes, one to bacteriocins, one to lasso peptides, two to the class of lanthipeptides, one to nonribosomal peptides, one to others, one to the class of type III polyketide synthases and one gene cluster belonging to the class of siderophores (**Fig. 4a**). For *Serratia* the *in silico* analysis revealed in total 9 gene clusters from which two gene clusters belonged to the class of Non-Ribosomal Peptides, one to Hsr-lactones, one to Aryl polyene- Siderophores, one to the class of T1PKS-NRPS hybrids, one to the class of Thiopeptides, one to the class of Butyrolactones, one to the class of Terpenes and one to the class of others (**Fig. 4b**). Flor *H. gracilis* the AntiSMASH analysis revealed that *H. gracilis* possesses relatively few gene clusters related to secondary metabolism. A total of three gene clusters for *H. gracilis* were detected, of which one belonged

to the class of bacteriocins, one to the class of terpenes, and one to aryl polyenes, the latter being a homolog to the aryl polyene gene cluster from *Xenorhabdus doucetiae* (Genbank: NZ_FO704550.1) **Fig. 4c**.

Pathway analysis in *H. gracilis* compared to *Serratia* and *Paenibacillus*

The RAST comparison of *Paenibacillus* and *H. gracilis* revealed that 504 unique enzymes (according to their EC numbers) were exclusive to *Paenibacillus*, while 434 were present only in *H. gracilis*; 532 EC numbers shared by both genomes (**Fig. 5a**). The RAST comparison of *Serratia* and *H. gracilis* revealed that 751 enzymes were present only in *Serratia*, and 260 were present only in *H. gracilis*. 727 EC numbers participating in diverse metabolic pathways were found in both genomes (**Fig. 5b**).

The RAST analysis revealed five missing genes in *H. gracilis* compared to *Serratia* and *Paenibacillus*. The missing genes were annotated with the following ontology terms: GO:0008473 (ornithine cyclodeaminase activity), GO:0008696 (4-amino-4-deoxychorismatelyase activity), GO:0003920 (GMP reductase activity), GO:0004035 (alkaline phosphatase activity) and GO:0008442 (3-hydroxyisobutyrate dehydrogenase). We verified if the loss of these genes would render specific pathways obsolete. However, alternative pathways routes are present for these GO terms according to the KEGG database. The pathway analysis by RAST did not suggest any essential missing pathway components in metabolic pathways in *H. gracilis*. Still, the comparison of the number (n) of genes present in each RAST subsystem category revealed major differences in several subsystem categories, specifically in the categories "Carbohydrates metabolism" and "Phosphorus metabolism" (**Fig. 5c**). *H. gracilis* possesses no genes for these categories, whereas *Paenibacillus* possesses 393 and 82 genes, and *Serratia* 395 and 46 genes, respectively. A major difference in the absolute number of genes in a category is also observed for Amino Acids and Derivatives, for which *H. gracilis* possesses 318 genes, *Paenibacillus* possesses 358 and *Serratia* 448 genes.

Effect of inter-specific interactions on gene expression

RNA- sequencing was performed to better understand the effects of bacterial interaction on gene expression of each interacting partner. The transcriptome analysis of monocultures and interspecies interactions revealed a total of 277 significant differentially expressed genes; 100 down-regulated and 177 up-regulated (**Supplementary Table 2**).

Effect of inter-specific interactions on gene expression in Paenibacillus and H. gracilis

In *Paenibacillus* histidine biosynthesis and dephosphorylation genes were up-regulated (**Supplementary Table 3**), while cellular-growth-related genes were down-regulated (**Supplementary Table 3**) at day 10 of the interaction with *H. gracilis* (**Fig. 6b**). For the interaction of *H. gracilis* with *Paenibacillus* a total of 15 significant differentially expressed genes were found (0 at day 5 and 15 at day ten). At day five, genes related to sulfur assimilation, chemotaxis and response to (chemical/external) stimuli were upregulated in *H. gracilis* in the presence of *Paenibacillus*. Genes related signal transduction (T) were the category with the most differentially expressed genes during the interaction of *H. gracilis* with *Paenibacillus* compared to the monoculture of *H. gracilis* (**Supplementary Table 8** and **9**, **Fig. 6a, b**).

Effect of inter-specific interactions on gene expression Serratia and H. gracilis

During the interaction of *Serratia* with *H. gracilis*, 61 genes were significantly differentially expressed at day five and 10 at day ten. At the day five, iron-sulfur cluster-assembly-related genes, a sulfur transferase and a transaminase were up-regulated, while genes related to inorganic diphosphatase activity, exonuclease activity and DNA repair were downregulated. At day ten, genes related to sulfur transmembrane transport, sulfur compound catabolism and cysteine biosynthesis were upregulated, and genes related to sulfur compound metabolism and translation were downregulated. (**Supplementary Table 4** and **5**). For *Serratia*, genes related to signal transduction and translation, ribosome structure and biogenesis were the most differentially expressed gene categories (**Fig. 6c**). For *H. gracilis* in interaction with *Serratia*, 182 differentially expressed genes were identified at day ten and only one at day five. At day five, genes related to the ribosome/ribonucleoproteins, organelle organisation/assembly and (iron)-sulfur cluster assembly were upregulated and genes related to the innate immune response (Toll Like Receptor signalling) were downregulated (**Supplementary Table 6** and **7**). At day ten, genes related to signal transduction and chemotaxis were up- regulated in *H. gracilis*. For *H. gracilis*, the most upregulated genes were linked to the chemotaxis pathway and iron scavenging, indicating activity in competition (**Fig. 6a**).

Metabolomic analysis of volatile compounds

The volatile blend composition of the monocultures differed from that of the co-cultures. Clear separations between the controls, monocultures and co-cultures were obtained in PLS-DA score plots (**Fig. 7a**). The analysis revealed a total of 25 volatile organic compounds produced by mono- and co-cultured bacteria that were not detected in the non-inoculated controls (**Table 2**).

Of these, 17 are identified and categorized in six chemical classes (alkenes, benzoids, sulfides, thiocyanates, terpenes, furans). The remaining eight compounds could not be assigned with certainty to a known compound. The most abundant volatile organic compounds were sulfur-containing compounds such as dimethyl disulfide ($C_2H_6S_2$) and dimethyl trisulfide ($C_2H_6S_3$). These two sulfur compounds were produced by all three bacteria. Interestingly an unknown compound with RT 26.4 min produced by the monocultures of *H. gracilis* was not detected in the interactions with *Serratia* (**Table 2**). Two other unknown compounds with RT 4.15 min and with RT 24.34 min produced by the monocultures of *Paenibacillus* were not detected in the interactions with *H. gracilis* (**Table 2**).

DART-MS based metabolomics

The metabolomics analysis based on DART-MS revealed separations between the controls, monocultures, and co-cultures as presented in PLS-DA score plots (**Fig. 7b**). The metabolomic composition of the monocultures differed from that of the co-cultures (**Fig. 7b**). Statistical analysis (ONE-WAY ANOVA and post-hoc TUKEY HSD-test) revealed 617 significant mass features present on day five and day ten of which 48 could be tentatively assigned to specific compounds. Most of the significant peaks were found in the co-cultures of *H. gracilis* with *Paenibacillus*. The significant mass features and the corresponding tentative metabolites can be found in **Supplementary Table 10**.

Mass spectrometry imaging metabolomics

LAESI-MSI was performed to visualize the localization of metabolites in their native environments in monoculture as well as during interaction without performing any extraction. Across all treatments, clear separation was observed amongst the samples for controls, monocultures and interactions **(Fig. 8a)**. An average of 1050 mass features was detected per treatment. To list mass features that could explain separation amongst the controls, monocultures and interactions, values of variable importance in projection (VIP) were calculated. The top 40 statistically significant mass features with a VIP score > 2.0 are shown in **Fig. 8b**. The mass features have been listed based on their decreasing influence on classification amongst the different treatments. To visualize the statistically significant mass features between monoculture and interaction samples in a pairwise manner, volcano plots were constructed **(Fig. S3)**. Metabolites with a log2 fold change threshold of 1.0 on the x-axis and a t-test threshold (*p* value) of 1.0 on the y-axis were considered significantly differentially abundant between monoculture and interaction samples.

The volcano plot (**Fig. S3a**) for *Hylemonella* monoculture (HM) and the interaction of *H. gracilis* with *Paenibacillus* (PH) shows 53 mass features (in green) located in the upper right quadrant, indicating that their concentrations are significantly higher in HM as compared to PH. 18 mass features (in red) in the upper left quadrant of the plot have a significantly lower concentration in HM as compared to PH. The box-and-whisker plots for the four statistically significant differentially abundant metabolites selected from the volcano plot for the pair HM and PH are shown in **Fig. S2a**. The ion intensity maps for these statistically significant metabolites are shown alongside box-and-whisker plots. The ion intensity maps are color coded based on the standard rainbow color scale where a pixel in red represented a high concentration and the pixel in black represents no concentration of the selected metabolite. As can be seen, *m/z* 425.2886 and *m/z* 558.2832 show higher abundance in interaction sample PH, whereas *m/z* 410.8587 and *m/z* 716.7610 display high abundance in HM as compared to PH.

For the pairwise analysis performed for *Paenibacillus* monoculture (PM) and the interaction of *H. gracilis* with *Paenibacillus* (PH), 149 mass features (in green) displayed significantly high concentration in PM and 75 mass features (in red) had significantly low concentration in PM as compared to PH (**Fig. S3b**). This is also evident in the box-and-whisker plots and the ion intensity maps that are presented for four statistically significant metabolites belonging to this set (**Fig. S2b)**.

For the pairwise analysis for *H. gracilis* monoculture (HM) and the interaction of *Serratia* and *H. gracilis* (SH), 57 mass features (in green) displayed significantly high concentration in HM and 42 mass features had significantly low concentration in HM as compared to SH (**Fig. S3c**). The box-and-whisker plots along with the ion intensity maps for four statistically significant metabolites belonging to this set are shown in **Fig. S2c**. For the pairwise analysis for *Serratia* monoculture (SM) and the interaction of *Serratia* and *H. gracilis* (SH), 135 mass features (in green) displayed significantly high concentration in SM and 65 mass features had significantly low concentration in SM as compared to SH (**Fig. S3d**). The box-and-whisker plots along with the ion intensity maps for four statistically significant metabolites belonging to this set are shown in **Fig. S2d**.

To visualize the number of shared and unique metabolites amongst the monoculture and interaction samples Venn diagrams were plotted. The Venn diagram (**Fig. S3e**) for monocultures *H. gracilis* and *Paenibacillus* and their interaction shows 80 metabolites unique

to *H. gracilis* monoculture, 75 metabolites unique to *Paenibacillus* monoculture and 100 metabolites that are unique during their interaction. 1062 metabolites were shared within these three treatments. Similarly, the Venn diagram **(Fig. S3f)** for monocultures *H. gracilis* and *Serratia* and their interaction shows 196 metabolites unique to *H. gracilis* monoculture, 48 metabolites unique to *Serratia* monoculture and 120 metabolites that are unique during their interaction.

Discussion

Here we report the first time isolation of *H. gracilis* from a terrestrial soil sample. This bacterium passed a 0.1 µm filter, which suggests a very small cell size, justifying ultra-small bacteria (26). However, against our expectation, the microscopical analysis revealed that this bacterium is not ultra-small in cell size but possesses a very thin diameter and showed the typical spiraled morphology known for these species (63-66). These observations are in line with previous research by Wang et al. showing that *H. gracilis* is capable of passing through filters of various pore sizes ranging from 0.45 µM to 0.1µM (67), most probably thanks to their cell shape and cell morphology.

The bacterial interaction assays revealed that H. gracilis grows better when interacting with Paenibacillus sp. Or Serratia. H. gracilis cell numbers were higher when exposed to cell-free supernatants of Paenibacillus and Serratia, suggesting that H. gracilis interaction benefits are related to the metabolites released by these bacteria. We hypothesized that H. gracilis grows better in co-culture, either because growth is stimulated by signals produced by the other organism, or because the environment that is created by the other organism allows H. gracilis to make more efficient use of the metabolic pathways. Indeed, the metabolic experiments with BioLog™ plates showed that during interspecific interactions of H. gracilis with Paenibacillus or with Serratia, more carbohydrates could be utilized compared to the monocultures. This is an interesting observation, as it indicates that the interaction of bacteria can trigger the production of exo-enzymes enabling the degradation of carbohydrates, which the bacteria were not able to degrade in monoculture.

We speculated that since *H. gracilis* grows better in interaction with other bacteria and is of relatively small cell size, it is possible that *H. gracilis* has evolved a genome streamlining strategy, i.e. the adaptive loss of genes for which functions it relies on interaction with other bacteria. Indeed, whole-genome sequencing of *H. gracilis* revealed a genome size of 3.82 Mbp. This is a relatively small genome size for free-living soil bacteria that typically have estimated average genome sizes of ~4.7 Mbp (34, 68-71). The *in silico* antiSMASH (44) comparison of

genes that are part of secondary metabolite gene clusters showed that the *H. gracilis* genome contained only three gene clusters encoding the production of secondary metabolites (bacteriocins, terpenes, and aryl polyenes), which provides additional evidence of genome streamlining. Terpenes and aryl polyenes are known as protective compounds against abiotic stressors, while bacteriocins have antimicrobial activities against closely related bacteria (17, 72-76). We hypothesized that *H. gracilis* has undergone genome streamlining, to be more competitive, by retaining only the most essential metabolic functions. Genome streamlining refers to the reduction of genome size by loss of genes, which may for instance evolve in symbiotic or co-occurring species when some genomic functions are redundant in one species and are functionally compensated by other species (Giovannoni et. al., 2014). Thus, gene loss and reduced genome size may cause dependency on other microbes in their surroundings, and this may explain a considerable part of the phenomenon of the uncultured microbial majority. Interestingly, most of the data supporting streamlining theory come from the study of bacteria from aquatic environments, where bacterial cell density is lower. However, the same process may be important in high density soil microbial communities.

To understand the mechanisms of interaction, we performed transcriptome analysis on the interaction pairs of *H. gracilis* with *Serratia* and *Peanibacillus*. Interestingly, more significantly differentially expressed genes were induced by *H. gracilis* in the other two competing bacteria as compared to the transcriptomic changes in *H. gracilis* induced by *Serratia* or *Paenibacillus* (**Supplementary Table 2**). Several processes, enriched according to GO term enrichment analysis, could be part of a mechanism(s) mediating interactions between *H. gracilis* and *Serratia* and *Paenibacillus*, for example genes related to chemotaxis. Moreover, the GO terms for signal transduction, secondary metabolite production and, cell motility were enriched in the transcriptome of *H. gracilis* during the interaction with *Paenibacillus*, suggesting that chemotaxis is an important feature during interspecific interactions between these two bacterial taxa (77, 78). In addition, Iron-sulfur (Fe-S) complex assembly GO terms were enriched in the transcriptomes of *H. gracilis* during the interaction with *Serratia* and *Paenibacillus*. Fe-S clusters are important for sustaining fundamental life processes: they participate in electron transfer, substrate binding/activation, iron or sulfur storage, regulation of gene expression, and enzyme activity (80, 81). This up-regulation could indicate that in co-culture, the interacting bacteria releases metabolites that *H. gracilis* could use for synthesizing Fe-S complexes. It is also possible that iron-sulfur complex assembly is activated during competition with the interacting bacteria for sulfur, or iron collection (82-85).

The metabolic pathway analysis showed that the loss of genes in *H. gracilis* does not appear to have resulted in functional loss of metabolic pathways. However, shedding non-necessary redundant genes in several metabolic pathways could explain why and how the genome of *H. gracilis* has become so small. Nevertheless, the lost genes are not essential to complete metabolic pathways and only appear to result in limited options in certain metabolic pathways. The RAST analysis showed that all metabolic pathways remain feasible. The only exception is EC term 5.2.1.1 (maleate isomerase) which makes it not clear which alternative pathway can be used by *H. gracilis* to synthesize fumarate. There are several ways to synthesize fumarate, e.g. in the glycolysis pathway (66, 92, 93) and in the citric acid cycle (66, 94). The performed metabolomics analysis revealed the production of specific antimicrobial compounds such as pyrollnitrin (*Serratia*) and 2,5-bis(1-methylethyl)-pyrazine (*Paenibacillus*) which are well known for their broad-spectrum antimicrobial activity (95-99). However, the produced antimicrobial compounds didn't show activity against *H. gracilis*: in both interactions, *H. gracilis* showed increased growth when growing in co-culture with either *Paenibacillus* or *Serratia*.

The understanding of natural metabolites that mediate interactions between organisms in natural environments is the key to elucidate ecosystem functioning. The detection and identification of the compounds that mediate such interactions is still challenging. Techniques such as mass spectrometry imaging (MSI) provide new opportunities to study environmentally relevant metabolites in their spatial context (35-37). In this study, the metabolomics was performed using three independent approaches namely DART-MS analysis, GC/MS-Q-TOF analysis and, ambient imaging mass spectrometry (LAESI- MS) from living bacterial colonies. The Imaging MS analysis revealed that several mass features were detected in higher abundance during the interaction of *H. gracilis* with *Paenibacillus*, these mass features were m/z 425.2886 and m/z 558.2832. However, the here used technology (LAESI-MSI) is not suitable for unambiguous compound annotation, but LAESI- MSI can still be used for putative compound annotation. To annotate the detected mass features to compounds with high certainty, LAESI mass spectrometry imaging should be coupled with ion mobility separation as suggested by (100-102). Yet, LAESI-MSI can help to spatially distinguish the produced secondary metabolites of living bacterial colonies with limited sample preparation and can give insight into the spatial distribution of metabolites.

Several studies indicate that the volatile composition of the volatiles greatly depends on biotic interactions and on growth conditions (15, 19, 59, 103, 104). Here, a higher number of volatile compounds were detected in the bacterial interaction pairs, most likely due to the combination

of emitted volatiles of the interacting bacteria. The high amount of sulfur-containing compounds indicates that these compounds are commonly produced by bacteria and might play an important role in signaling during interspecific interactions (105, 106). However, no novel volatile compounds during the co-cultivation of the three bacteria were detected.

Overall, our study showed that *H. gracilis* is able to pass through 0.1 µM filter, and is present in terrestrial environments. The growth and behavior of *H. gracilis* were dependent on the interacting partner and they might be metabolically dependent on the neighboring bacteria. At the same time, *H. gracilis* is able to change the behaviour of the interacting bacteria without direct cell-cell contact. This study have laid a good bases for isolating soil bacterial with a small cell size and for exploring interactions between bacteria with different cell and genome size. Deciphering such interactions is key to understanding ecosystem functioning and the assembly of microbial communities.

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Author contributions

OT and PG designed the experiments. OT, AO, PK and WIJ performed the lab experiments. OT, PK and performed the data analysis and prepared the figures and tables. OT, PK, AO, VT, MHM, PB, KJFV and PG wrote the manuscript.

Figure legends

Figure 1: Microscopy pictures of *Hylemonella gracilis* (**a**) colonies on agar plates captured at 20x magnification and (**b**) single bacterial cells captured at 400 X magnification.

Figure 2: Colony counts (CFU/mL) revealed after 5 days of incubation during the plate based interaction experiment (a) and (b) revealed during the cell free supernatant (CFS) exposure experiment. Abbreviations: *H. gracilis* monoculture (HM), *Paenibacillus* sp. AD87 monoculture (PM), *Paenibacillus* sp. AD87 – *H. gracilis* coculture (PH), *S. plymuthica* monoculture (SM), *S. plymuthica* - *H. gracilis* coculture (SH). *H. gracilis* - *Paenibacillus* sp. AD87 coculture (HP), *H. gracilis* – *S. plymuthica* coculture (HS). Significant differences in

cell counts (CFU/mL) between co-cultures (treatment) and monocultures (controls) are indicated by asterisks (ONE-WAY ANOVA, post-hoc TUKEY test).

Figure 3: Results overview of the Biolog EcoPlate™ experiment. The EcoPlate contains 31 different carbon sources, bacteria were inoculated in monoculture or in pairwise combinations. Colour codec: blue=carbon source could be utilized in monoculture, yellow= carbon source could be utilized in co-culture. Abbreviations: *Paenibacillus* sp. AD87 monoculture (PM), *Paenibacillus* sp. AD87 - *H. gracilis* coculture, (PH) *H. gracilis* monoculture (HM), *S. plymuthica* PRI-2C monoculture (SM), *S. plymuthica* PRI-2C - *H. gracilis* coculture (SH).

Figure 4: *In silio* comparison of biosynthetic gene clusters (BGCs) present in the three soil bacteria based on antismash *in silico* analysis (https://antismash.secondarymetabolites.org/). From left to right (**a**) *Paenibacillus* sp. AD87 with a genome size of 7.0 MBp, n=10 gene clusters for secondary metabolites, (**b**) *S. plymuthica* PRI-2C with a genome size of 5.4 MBp, n=9 gene clusters for secondary metabolites and (**c**) *H. gracilis* with a genome size of 3.8 MBp, n= 3 gene clusters for secondary metabolites.

Figure 5: Results of the comparison analysis carried out with the RAST pipeline (https://rast.nmpdr.org/) (**a**) Venn- diagram showing the results for the number (n) of expressed genes present solely in monoculture and the number of shared expressed genes for the monocultures of *Hylemonella* (HM), *Paenibacillus* monoculture (PM) and *Serratia* monoculture (SM) and the interaction of *Hylemonella* with *Paenibacillus* (PH) and for the interaction of *Hylemonella* with *Serratia* (SH). (**b**) Boxplot showing number (n) of expressed genes present in each RAST subsystem category.

Figure 6: Pie-charts representing up-regulated genes identified by differential gene expression analysis and COG annotation (**a**) *Hylemonella gracilis* monoculture gene expression level (**b**) *H. gracilis* in co-culture with *Paenibacillus* sp. AD87; (**c**) *H. gracilis* co-culture with *S. plymuthica* PRI-2C. In the co-culture of *H. gracilis* with *Paenibacillus* sp. AD87, genes related to signal transduction (**T**) were the category with the most differentially expressed genes. In the co- culture of *H. gracilis* with *S. plymuthica* PRI-2C genes related to signal transduction (**T**), translation, ribosome structure and biogenesis (**J**) were the most prevalent differentially expressed gene categories.

Figure 7: PLSD-A plots of the metabolomics data (**a**) PLS-DA 2D- plots of volatiles emitted by monocultures and pairwise combinations of *H. gracilis*, *Paenibacillus* and *Serratia plymuthica* after ten days of inoculation, time point (t=10 days) (**b**) PLS-DA 2D- plots of the analysed DART-MS data of monocultures and mixtures of *H. gracilis*, *Paenibacillus* sp. AD87 and *Serratia plymuthica* PRI-2C after ten days of inoculation, time point (t=10 days).

Figure 8: PLS-DA plots of the first 40 significant mass features observed after analysis of the LAESI-MSI data. (a) PLS-DA score plot for *H. gracilis* monoculture (HM), *Paenibacillus sp. AD87* monoculture (PM), *Paenibacillus* sp. AD87 – *H. gracilis* coculture (PH), *S. plymuthica* PRI-2C monoculture (SM), *S. plymuthica* PRI-2C - *H. gracilis* coculture (SH) and TSBA control (TSBA). **(b)** First 40 statistically significant features identified by PLS-DA based on Variable Importance in Projection (VIP) score. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.

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Table 1: Genome assembly statistics and outcome of the *in silico* analysis on secondary metabolite gene clusters of *H. gracilis, Serratia plymuthica* PRI-2C and *Paenibacillus* sp. AD87.

Feature / Organism	***Hylemonella gracilis***	***S. plymuthica* PRI-2C**	***Paenibacillus* sp. AD87**
contigs	1	1	30
bases	3822245	5474685	7086713
number of chromosomes	1	1	1
size chromosome 1	3822245	5464425	7086713
CDS	3648	4929	6216
GC- content (%)	65.1	55.7	46.2
number of RNAs	53	109	146
genes	3625	5284	6375
in silico detected secondary metabolite clusters (antiSMASH)	3	9	10
Total genome size (bases)	**3822245**	**5474685**	**7086713**

Table 2: Tentatively identified volatile organic compounds (VOCs) produced by a *H. gracilis*, *Serratia plymuthica* PRI-2C and *Paenibacillus* sp. AD87 strains in mono- and co-cultures.

						Detected in treatment				
#	Compound name	RT*	ELRI**	p-value***	chemical class	HM	SM	SH	PM	PH
1	2-methylfuran	2,18	738	0,041	Furan	X		X	X	X
2	2-methylpropanoic Acid	3,01	755	0,014	Alkenes	X	X	X	X	X
3	mix pentanal + heptane	3,21	760	0,008	Alkenes	X		X		X
4	methyl thycocyanate	3,44	764	0,020	Thioesters	X	X	X	X	X
5	1-Pentanol	3,95	772	0,012	Alkenes		X	X	X	X
6	dimethyl disulfide	4,01	775	0,012	Sulfides	X	X	X	X	X
7	unknown compound 1	4,15	778	0,003	-		X	X	X	
8	toluene	4,44	784	0,014	Benzenoids	X	X	X	X	X
9	methyl Isovalerate	4,76	789	0,018	Terpenes		X	X	X	X
10	cyclohexane	8,07	852	0,031	Alkenes		X	X	X	X
11	dimethyl trisulfide	11,35	914	0,013	Sulfides	X	X	X	X	X
12	benzonitrilie	12,06	928	0,037	Alkenes	X	X	X	X	X
13	2-Ethyl-4-methylpentan-1-ol	17,26	1026	0,015	Alkenes		X	X	X	X
14	2,5-bis(1-methylethyl)-pyrazine	20,56	1090	0,031	Pyrazines				X	X
15	undecane	21,31	1100	0,014	Alkenes	X		X	X	X
16	unknown compound 2	24,34	1140	0,013	-				X	
17	unknown compound 3	25,92	1160	0,011	-	X	X	X	X	X
19	unknown compound 4	26,40	1165	0,018	-	X		X	X	X
20	unknown compound 5	26,90	1170	0,003	-	X			X	X
21	alpha-terpineol	27,34	1178	0,016	Terpenes	X	X	X	X	X
22	undecane, 2,6-dimethyl	28,27	1190	0,004	Benzenoids	X	X	X	X	X
23	gamma-terpineol	28,42	1192	0,006	Terpenes	X		X		
24	terpene like compound 1	29,32	1202	0,012	Terpenes	X		X		
25	terpene like compound 2	31,49	1231	0,009	Terpenes	X	X	X	X	X
Number of detected compounds (n)						**16**	**15**	**20**	**20**	**19**

Abbreviations:

HM= *H. gracilis* monoculture, SM= *Serratia plymuthica* PRI-2C monoculture, PM= *Paenibacillus* sp. AD87 monoculture, PH= *Paenibacillus* sp. AD87 and *H. gracilis* co-culture, SH= *Serratia plymuthica* PRI-2C and *H. gracilis* co-culture

= Compound number

RT* = Retention time, the RT value stated is the average of three technical replicates.

ELRI** = Experimental linear retention index value, the RI value stated is the average of three replicates.

p-value***= statistical significance (peak area and peak intensity)

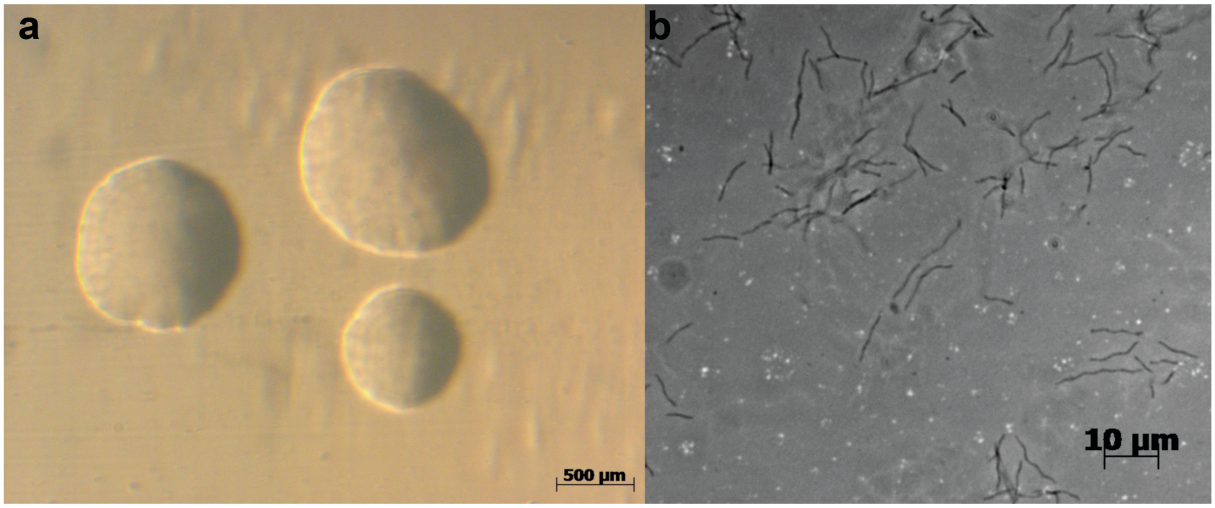

Figure 1: Microscopy pictures of *Hylemonella gracilis* (**a**) colonies on agar plates captured at 20x magnification and (**b**) single bacterial cells captured at 400 X magnification.

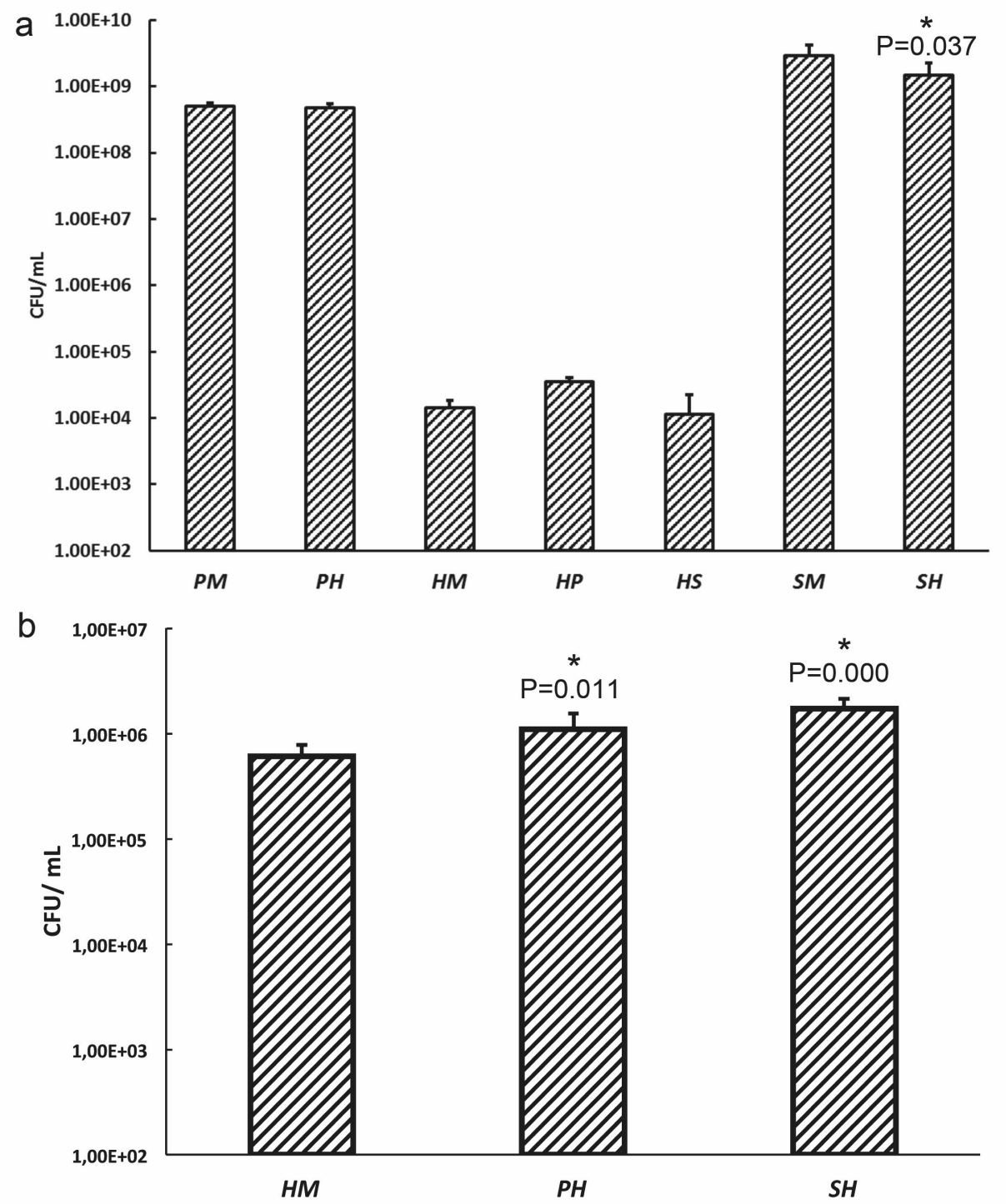

Figure 2: Colony counts (CFU/mL) revealed after 5 days of incubation during the plate based interaction experiment (a) and (b) revealed during the cell free supernatant (CFS) exposure experiment. Abbreviations: *H. gracilis* monoculture (HM), *Paenibacillus* sp. AD87 monoculture (PM), *Paenibacillus* sp. AD87 – *H. gracilis* coculture (PH), *S. plymuthica* monoculture (SM), *S. plymuthica* - *H. gracilis* coculture (SH). *H. gracilis* - *Paenibacillus* sp. AD87 coculture (HP), *H. gracilis* – *S. plymuthica* coculture (HS). Significant differences in cell counts (CFU/mL) between co-cultures (treatment) and monocultures (controls) are indicated by asterisks (ONE-WAY ANOVA, post-hoc TUKEY test).

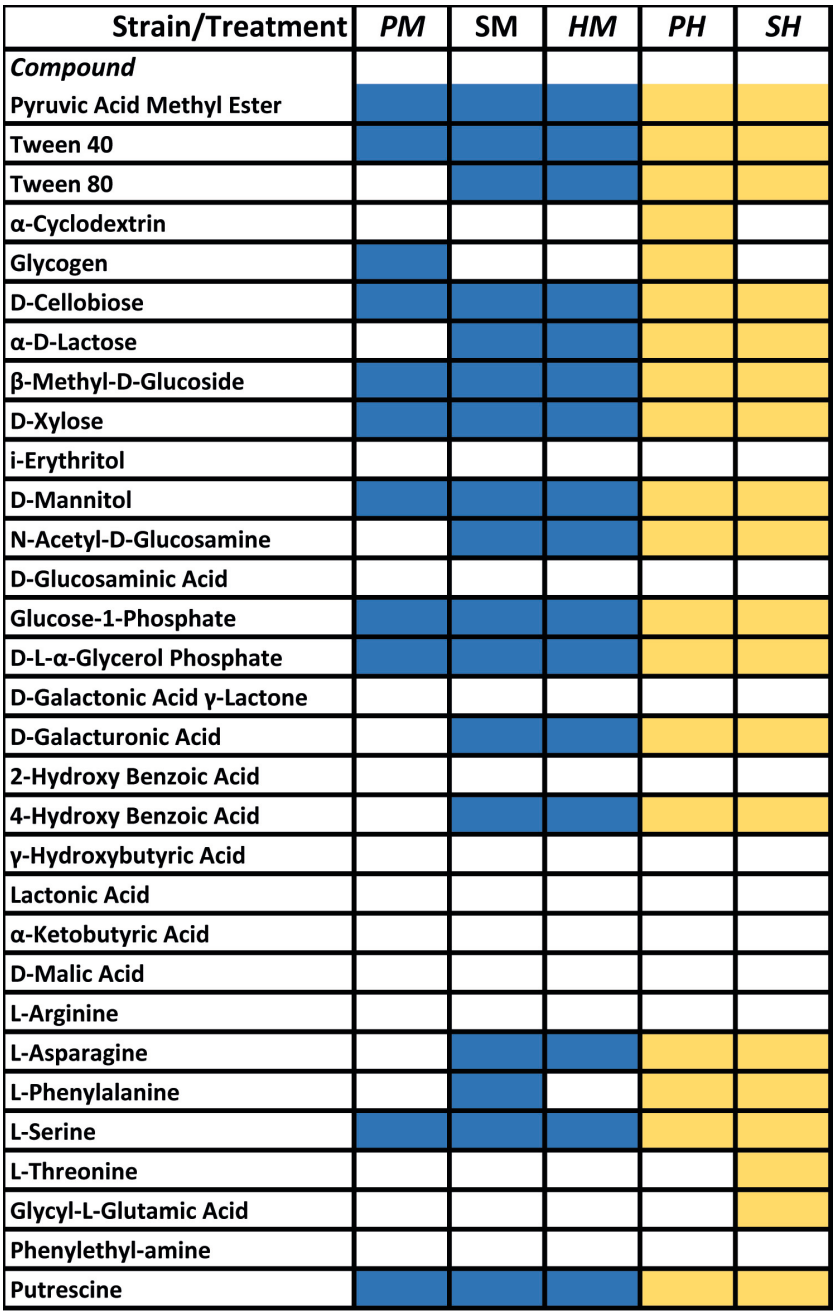

Figure 3: Results overview of the Biolog EcoPlate™ experiment. The EcoPlate contains 31 different carbon sources, bacteria were inoculated in monoculture or in pairwise combinations. Colour codec: blue=carbon source could be utilized in monoculture, yellow= carbon source could be utilized in co-culture. Abbreviations: *Paenibacillus* sp. AD87 monoculture (PM), *Paenibacillus* sp. AD87 - *H. gracilis* coculture, (PH) *H. gracilis* monoculture (HM), *S. plymuthica* PRI-2C monoculture (SM), *S. plymuthica* PRI-2C - *H. gracilis* coculture (SH).

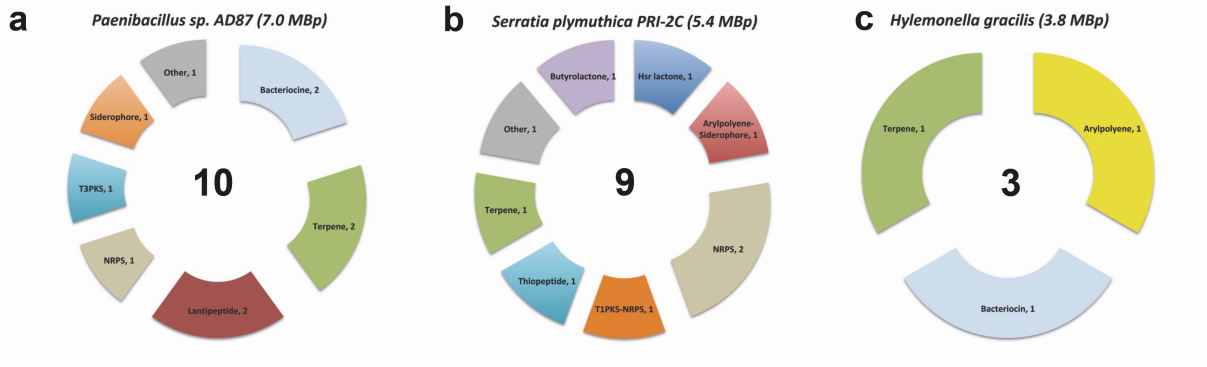

Figure 4: *In silio* comparison of biosynthetic gene clusters (BGCs) present in the three soil bacteria based on antismash *in silico* analysis (https://antismash.secondarymetabolites.org/). From left to right (**a**) *Paenibacillus* sp. AD87 with a genome size of 7.0 MBp, n=10 gene clusters for secondary metabolites, (**b**) *S. plymuthica* PRI-2C with a genome size of 5.4 MBp, n=9 gene clusters for secondary metabolites and (**c**) *H. gracilis* with a genome size of 3.8 MBp, n= 3 gene clusters for secondary metabolites.

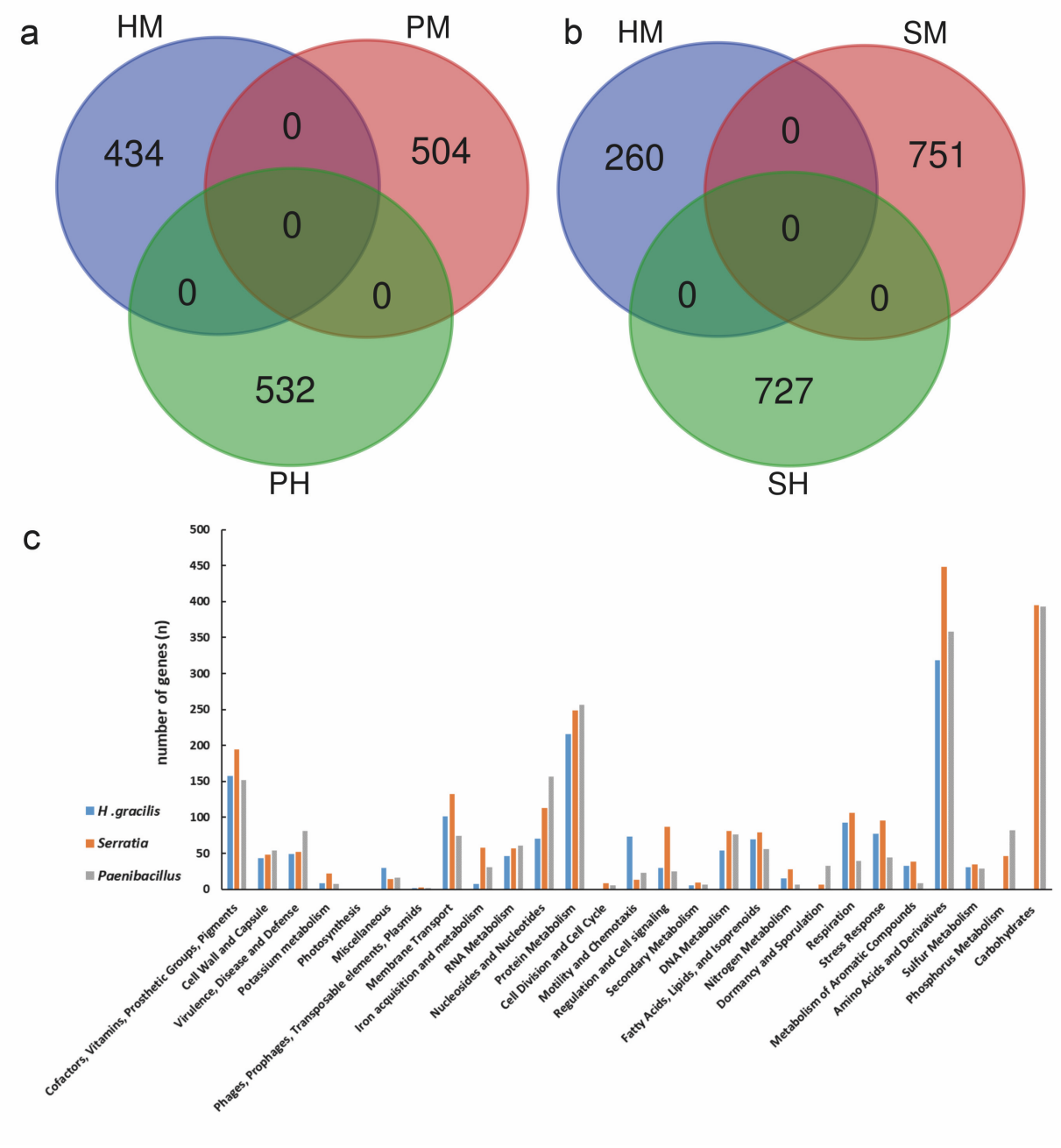

Figure 5: Results of the comparison analysis carried out with the RAST pipeline (https://rast.nmpdr.org/) (**a**) Venn- diagram showing the results for the number (n) of expressed genes present solely in monoculture and the number of shared expressed genes for the monocultures of *Hylemonella* (HM), *Paenibacillus* monoculture (PM) and *Serratia* monoculture (SM) and the interaction of *Hylemonella* with *Paenibacillus* (PH) and for the interaction of *Hylemonella* with *Serratia* (SH). (**b**) Boxplot showing number (n) of expressed genes present in each RAST subsystem category.

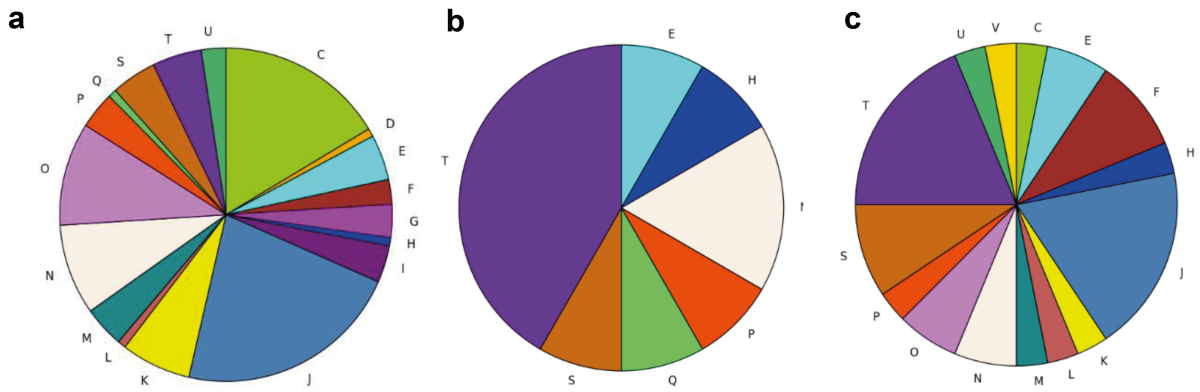

Figure 6: Pie-charts representing up-regulated genes identified by differential gene expression analysis and COG annotation (**a**) *Hylemonella gracilis* monoculture gene expression level (**b**) *H. gracilis* in co-culture with *Paenibacillus* sp. AD87; (**c**) *H. gracilis* co-culture with *S. plymuthica* PRI-2C. In the co-culture of *H. gracilis* with *Paenibacillus* sp. AD87, genes related to signal transduction (**T**) were the category with the most differentially expressed genes. In the co- culture of *H. gracilis* with *S. plymuthica* PRI-2C genes related to signal transduction (**T**), translation, ribosome structure and biogenesis (**J**) were the most prevalent differentially expressed gene categories.

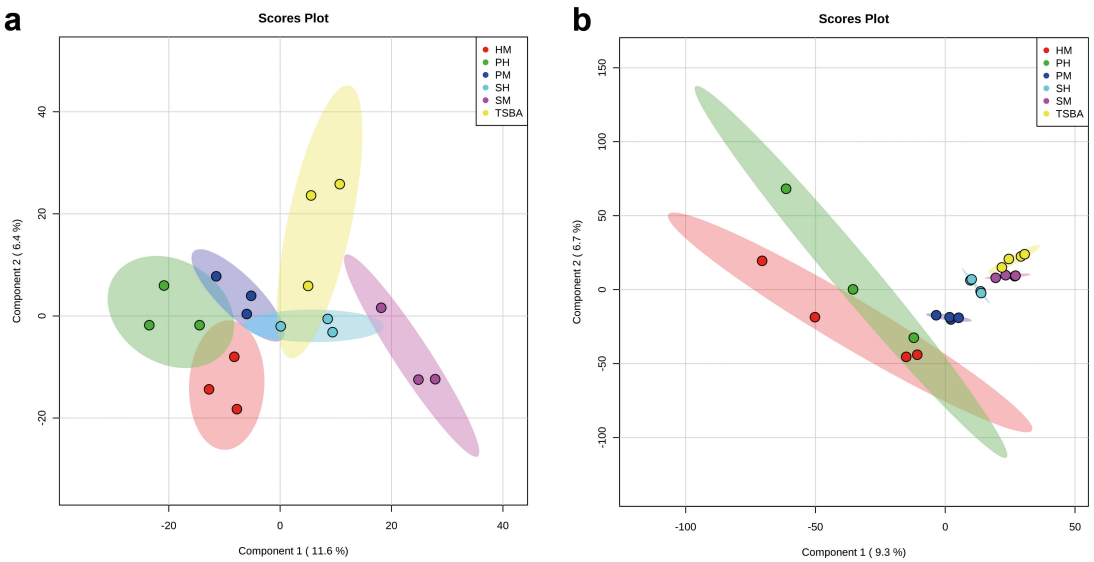

Figure 7: PLSD-A plots of the metabolomics data (**a**) PLS-DA 2D- plots of volatiles emitted by monocultures and pairwise combinations of *H. gracilis*, *Paenibacillus* and *Serratia plymuthica* after ten days of inoculation, time point (t=10 days) (**b**) PLS-DA 2D- plots of the analysed DART-MS data of monocultures and mixtures of *H. gracilis*, *Paenibacillus* sp. AD87 and *Serratia plymuthica* PRI-2C after ten days of inoculation, time point (t=10 days).

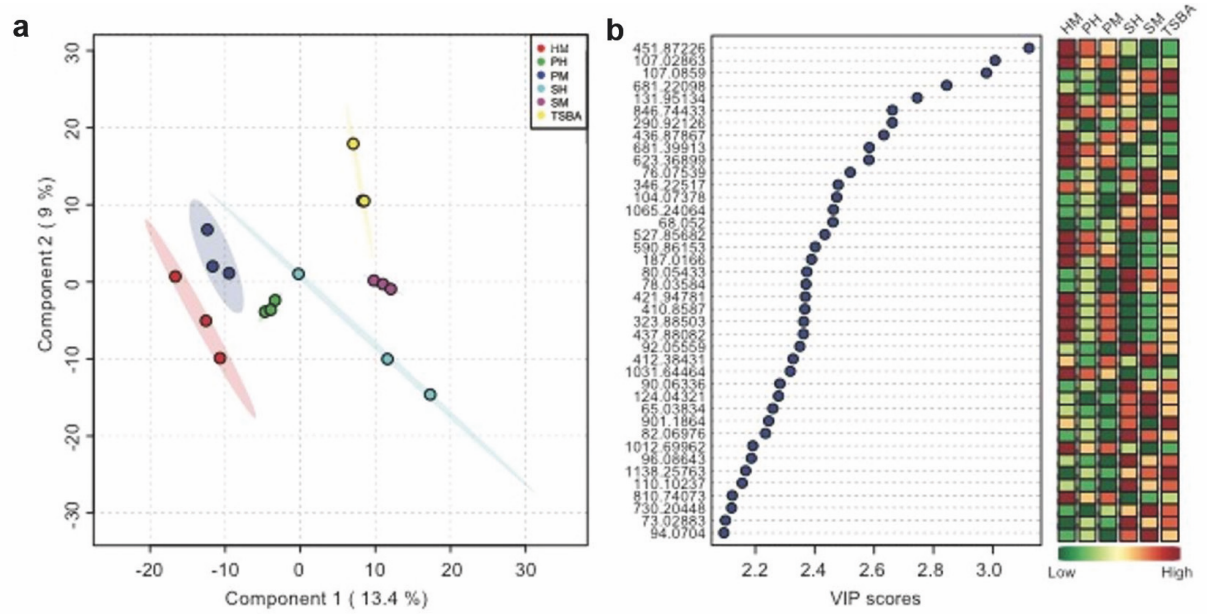

Figure 8: PLS-DA plots of the first 40 significant mass features observed after analysis of the LAESI-MSI data. (a) PLS-DA score plot for *H. gracilis* monoculture (HM), *Paenibacillus sp. AD87* monoculture (PM), *Paenibacillus* sp. AD87 – *H. gracilis* coculture (PH), *S. plymuthica* PRI-2C monoculture (SM), *S. plymuthica* PRI-2C - *H. gracilis* coculture (SH) and TSBA control (TSBA). **(b)** First 40 statistically significant features identified by PLS-DA based on Variable Importance in Projection (VIP) score. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.