Exploring the interspecific interactions and the metabolome of the soil isolate *Hylemonella gracilis*

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Abstract

Microbial community analysis of aquatic environments showed that an important component of its microbial diversity consists of bacteria with cell sizes of ~0.1 µm. Such small bacteria can show genomic reductions and metabolic dependencies with other bacteria. However, so far no study investigated if such bacteria exist in terrestrial environments like e.g. soil.

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*. A set of co-culture assays with phylogenetically distant soil bacteria with different cell and genome sizes was performed. The co-culture assays revealed that *H. gracilis* grows better when interacting with other soil bacteria like *Paenibacillus* sp. AD87 *and Serratia plymuthica*. Transcriptomics and metabolomics showed that *H. gracilis* was able to change gene expression, behavior, and biochemistry of the interacting bacteria without direct cell-cell contact.

Our study indicates that bacteria are present in the soil that can pass through a 0.1 µm filter. These bacteria may have been overlooked in previous research on soil microbial communities. Such small bacteria, exemplified here by *H. gracilis*, are able to induce transcriptional and metabolomic changes in other bacteria upon their interactions in soil. In vitro, the studied interspecific interactions allowed utilization of growth substrates that could not be utilized by monocultures, suggesting that biochemical interactions between substantially different sized soil bacteria may contribute to the symbiosis of soil bacterial communities.

Importance

Analysis of aquatic microbial communities revealed that parts of its diversity consist of bacteria with cell sizes of ~0.1 µm. Such bacteria can show genomic reductions and metabolic dependencies with other bacteria. So far, no study investigated if such bacteria exist in terrestrial environments e.g. soil. By applying a novel isolation method, we show that such bacteria also exist in soil. The isolated bacteria was identified as *Hylemonella gracilis*.

Co-culture assays with phylogenetically different soil bacteria revealed that *H. gracilis* grows better when co-cultured with other soil bacteria. Transcriptomics and metabolomics showed that *H. gracilis* was able to change gene expression, behavior, and biochemistry of the interacting bacteria without direct contact. Our study revealed that bacteria are present in soil that can pass through 0.1 µm filters. Such bacteria may have been overlooked in previous research on soil microbial communities and may contribute to the symbiosis of soil bacterial communities.

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 chemically modify their niche but also affect the behavior and the secondary metabolite production of nearby bacteria (9-11). Soil bacteria are known to produce a wide range of 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 physicochemical 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 densities compared to soil (10^3 - 10^6 cells/mL) (22-24). Recent studies have shown that a significant component of aquatic microbial diversity consists of bacteria with small cell sizes of about ~0.1 µm (25-27). However, little is known if bacteria with such cell sizes exist in soil environments, for instance in water-filled soil pores. One can assume that a small cell size can be an advantage

110 in challenging environments like soil. However, the distribution of microorganisms in soil is
111 influenced by its water and moisture content, and a low soil moisture content leads to lower
112 connectivity between soil pores, and thus to a lower number of accessible micro-habitats.

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

124 Here, we aimed to explore if bacteria that are able to pass through 0.1 µm filters are
125 present in soil, and if such bacteria are cultivable. We further investigated their interaction
126 with phylogenetically different bacteria commonly occurring in soil. The major research
127 questions were if, and how inter-specific interactions between bacteria that pass a 0.1 µm
128 filter and other common soil bacteria that cannot pass 0.1 µM filters affected their fitness,
129 behavior, gene expression, and the production of secondary metabolites.

130

131 ## Materials and Methods

132 **Isolation and identification of bacteria that pass through 0.1 µm filters**

133 **Isolation of *H. gracilis* from soil**

134 After removing the grassland vegetation, a topsoil core was collected and mixed, a sample of
135 10 g was suspended in 90 ml of 10 mM Phosphate-buffer (pH 6.5) and shaken with gravel (2-
136 4 mm) at 250 rpm for 45 min. The extract was filtered through sterile gauze pads and
137 subsequently through sterile Whatmann 1mm paper filter using Buchner funnel. Purified
138 extract was filtered again through a syringe filter 0.2 µM and afterwards through 0.1 µM filter
139 (GE-Healthcare). The filtered extract was plated on 1/10th TSBA plates immediately after
140 isolation and incubated at 25 °C (**Supplementary Figure 1**). The plates were inspected daily
141 using a stereomicroscope (Leica M205C) screening for bacteria colonies.

142 **Bacteria and culture conditions**

143 The bacterial strains used in this study are the Gram-negative strain *S. plymuthica* PRI-2C
144 (gamma-Proteobacteria) (35), the Gram-positive strain *Paenibacillus* sp. AD87 (Firmicutes)
145 (10, 36, 37) and the Gram-negative *H. gracilis* isolate NS1 (beta-Proteobacteria). The
146 bacterial isolates were pre-cultured from -80 °C glycerol stocks on 1/10th TSBA (38) or on
147 LB-A plates (*H. gracilis*) (LB-Medium Lennox, Carl Roth GmbH + Co. KG, 20 gL^{-1} Bacto
148 Agar) and incubated at 24 °C prior application. All bacterial isolates are listed in
149 **Supplementary Table 1.**
150
151 **Identification of *H. gracilis***
152 For the identification of *H. gracilis* 16S rRNA PCR was performed from grown colonies in a
153 50 µl PCR- GoTaq™ green master mix (Promega Corp. Madison, USA cat# M712). For 16S
154 rRNA gene amplification the following primers were used: forward primer 27f (5'- AGA
155 GTTT GAT CMT GGC TCAG -3'), reverse primer 1492r (5'- GRT ACC TTG TTA CGA
156 CTT -3'), amplifying ~1465 bp from the 16S rRNA gene (39, 40) (modified). All PCR
157 reactions were performed on a BIO-RAD C1000 Touch™ PCR machine (BIO-RAD,
158 Veenendaal, the Netherlands) with these settings: initial cycle 95 °C for 3 min. and 30 cycles
159 of 94 °C for 30 sec., 55 °C for 45 sec. and 72 °C for 1 min. and final extension at 72 °C for 5
160 minutes. The PCR products were purified using the Qiagen PCR purification kit and sent to
161 MACROGEN (MACROGEN Europe, Amsterdam, the Netherlands for 16S rRNA
162 sequencing.
163
164 **Microscopy**
165 Microscopy pictures of *H. gracilis* cells were taken at 400-fold magnification with an Axio
166 Imager M1 microscope (Carl Zeiss, Germany) under phase-contrast illumination with an
167 AxioCam MRm camera. Macroscopic colony pictures of *H. gracilis* were taken with an
168 OLYMPUS Binocular at 20 X magnification. Images were analyzed with AXIO VISION v4.7
169 (Carl Zeiss Imaging Solutions GmbH, Germany).
170
171 **Bacterial interactions assays**
172 After four-days of pre-culture, a single colony of *Paenibacillus* sp. AD87 and *S. plymuthica*
173 PRI-2C and *H. gracilis* was picked and inoculated in 20 mL 1/10th TSB (*Paenibacillus* sp.
174 AD87 and *S. plymuthica* PRI-2C) and grown overnight at 24 °C at 220 rpm. For the
175 inoculation of *H. gracilis* a single colony was picked from a TSBA plate and inoculated in 20
176 mL LB-medium and grown for 3 days at 24 °C, 200 rpm. For the interaction assay an

inoculation mix of each bacterial strain (*Paenibacillus* sp. AD87, *S. plymuthica* PRI-2C, *H. gracilis*) was prepared by diluting the bacterial isolates in 20 mL of 10 mM Phosphate-buffer (pH 6.5) to an OD_{600} of 0.005 (*Paenibacillus* sp. AD87 and *S. plymuthica* PRI-2C) or to an OD_{600} of 0.05 (*H. gracilis*), which corresponds to 10^5 CFU/mL. A droplet of 10 µl 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). All treatments were performed in triplicates on $1/10^{th}$ TSBA plates incubated at 24 °C. After the growth time of 3 days the bacteria were scratched and washed from the plates by using sterile cell scratchers and Phosphate buffer. For the enumeration of the cell counts (CFU/mL) dilution series of the scratched bacteria were prepared and plated in triplicates on $1/10^{th}$ TSBA plates and grown for 48 hours. Enumeration was carried out on an aCOlyte Colony Counter (Don Whitley Scientific, Meintrup DWS Laborgeräte GmbH**,** Germany).

Enumeration of growth inhibitory or growth promoting effects of cell-free supernatants of *Paenibacillus* sp. AD87and *S. plymuthica* PRI-2C on the growth of *H. gracilis*

A bacterial growth assay in liquid media supplemented with cell-free supernatants (CFS) of *Paenibacillus* sp. AD87 and *S. plymuthica* PRI-2C was conducted. For the assay single colonies of *Paenibacillus* sp. AD87, *S. plymuthica* PRI-2C and *H. gracilis* were inoculated in 20 mL $1/10^{th}$ TSB (*Paenibacillus* sp. AD87 and *S. plymuthica* PRI-2C) or in 20 mL LB-medium (*H. gracilis*) and grown overnight at 24 °C, 220 rpm or for three days (*H. gracilis*). For the preparation of *Paenibacillus* sp. AD87 and *S. plymuthica* PRI-2C cell-free supernatant (CFS) the grown cultures were centrifuged at 5000 rpm for 20 minutes (at room temperature) and filtered through 0.2 µM filters (GE Healthcare). For the assay, *H. gracilis* was inoculated into 20 mL liquid LB- media at an OD_{600} of 0.05. The growth media was then supplemented either with 20 % (v/v) CFS of *Paenibacillus* sp. AD87 or *S. plymuthica* PRI-2C or with 20 % (v/v) of filter sterilized liquid $1/10^{th}$ TSB media (control). The cultures were incubated at 24 °C at 220 rpm for 7 days and the bacterial growth was monitored by optical density (absorbance at 600nm) measurements and by plate counting. After five days of growth, the CFU/ml of *H. gracilis* grown in presence of CFS of *Paenibacillus* sp. AD87 or *S. plymuthica* PRI-2C were enumerated by plate counting. For this, the cultures were sampled and dilution series were prepared in triplicates and a volume of 100 µl of each serial dilution was plated in three replicates with a disposable Drigalski spatula on $1/10^{th}$ TSBA plates. CFU Enumeration was carried out on an aCOlyte Colony Counter (Don Whitley Scientific, Meintrup DWS Laborgeräte GmbH, Germany).

211 **DNA isolation and genome sequencing of *H. gracilis***

212 Genomic DNA of *H. gracilis* was extracted using a QIAGEN Genomic-tip 500/G DNA kit
213 Qiagen, cat# 10262 . Genome sequencing was performed on the PacBio RS II platform
214 (Pacific Biosciences, Menlo Park, CA, USA) using P6-C4 chemistry at the Institute for
215 Genome Sciences (IGS), Baltimore, Maryland, USA. The sequencing resulted in a total of
216 70,101 reads with N50 of 17 309 nucleotides. The PacBio raw sequences were analyzed using
217 SMRT portal V2.3.0.140936 p.4150482. Sequences were assembled *de novo* with the
218 RS_HGAP_assembly 3 software (Pacific Biosciences, Menlo Park, CA, USA) with default
219 settings on an estimated genome size of 3.8 Mbp. The resulting assemblies were subjected to
220 scaffolding using the RS_AHA_scaffolding 1 software. The genome assembly properties are
221 shown in **Table 1**. Final contigs were annotated using PROKKA V1.11 (41) and InterproScan
222 5.16 55.0 (42). The whole genome sequence was submitted as *Hylemonella gracilis* strain
223 NS1 to NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under accession #
224 CP031395.

225

226 ***In silico* analysis of secondary metabolite gene clusters**

227 For *in silico* analysis of secondary metabolite gene clusters, the genome sequences of *H.*
228 *gracilis, Paenibacillus* sp. AD87 and *S. plymuthica* PRI-2C were submitted to the
229 antiSMASH web server (http://antismash.secondarymetabolites.org/) version 4.0 (43).

230 **RNA isolation and sequencing**

231 Sampling for total RNA extractions was performed in triplicates after five and ten days of
232 incubation on bacteria grown on 1/10th TSBA plates either in co-culture or monoculture as
233 described previously (Bacterial interactions assays on 1/10th TSBA plates). For the isolation
234 of bacterial cell material a volume of 1 mL of 10 mM phosphate buffer (pH 6.5) was added to
235 the surface of the 1/10th TSBA plates and grown bacterial cells were suspended from the plate
236 surface with a disposable cell scratcher (VWR international B.V., the Netherlands). For total
237 RNA extraction the obtained cell suspension was transferred to a tube containing RNA
238 Protect Bacteria Reagent (Qiagen, cat# 76506) and centrifuged for 20 min. at 20,000g, 4 °C.
239 The supernatant was discarded and the resulting cell pellets were stored at -80 °C. Total RNA
240 was extracted using the Aurum Total RNA Mini Kit (BIO-RAD) according to the
241 manufacturer's protocol. Samples were treated with TURBO DNA free Kit (AMBION)
242 according to the manufacturer's protocol. The RNA concentration and quality was checked on
243 a NanoDrop Spectrophotometer (ND 2000, Thermo Fisher Scientific, the Netherlands) and on
244 a 1.0 % TBE agarose gel. Samples were subjected to RNA sequencing at the Erasmus Center

245 for Biomics (www.biomics.nl), Erasmus MC, Rotterdam, The Netherlands using the Illumina
246 HiSeq 2500 sequencing platform. The obtained reads were checked for quality using Fastq.
247 For the estimation of the transcripts, the filtered sequences were aligned against the cDNA
248 sequences of *H. gracilis*, *Paenibacillus* sp. AD87 and *S. plymuthica* PRI-2C using Bowtie 2
249 (2.2.5) (44) with the following settings: -- no-mixed -- no-discordant -- gbar 1000 – end-to-
250 end. Transcript abundance was calculated using RSEM V1.1.26 (45) and differential
251 expression between the treatments was calculated using edgeR V3.2 package in the R
252 environment (46-48).
253
254 **Pathway annotations**
255 Please see Supplementary Methods.
256
257 **Exploration of missing genes and genome streamlining in *Hylemonella***
258 RAST annotations of *S. plymuthica* PRI-2C, *Paenibacillus* sp. AD87 and *H. gracilis* were
259 used to compare their genomes and to explore the genomes for missing genes in metabolic
260 pathways (http://rast.nmpdr.org) (49-51). The missing gene sequences were extracted and
261 assigned with KEGG Orthology (52, 53). Presence/absence of genes belonging to metabolic
262 pathways was compared across the three genomes to identify shared genes and pathways and
263 to determine incomplete metabolic pathways in *H. gracilis*.
264
265 **Catabolic profiling**
266 To determine the carbon source usage abilities of *H. gracilis* and *Paenibacillus* sp. AD87and
267 *S. plymuthica* PRI-2C strains, Biolog EcoPlate (Labconsult S.A.- N.V., Bruxelles, Belgium)
268 assays were performed (54, 55). Bacteria were cultured in monoculture or in co-culture in
269 single wells of the Biolog EcoPlate™. A, single colony of each bacterial strain was picked
270 and inoculated in 15 mL 1/10th TSB or 15 mL LB- liquid media. Bacteria were grown
271 overnight (*Paenibacillus* sp. AD87 and *S. plymuthica* PRI-2C) or for two days (*H. gracilis*) at
272 24 °C at 250 rpm. Grown bacteria cultures were washed twice by centrifugation at 4.500 rpm
273 for 15 minutes at room temperature, the supernatant discarded and the pellet was washed and
274 re-suspended in 5 mL of 10 mM phosphate buffer. The re-suspended cultures were diluted to
275 an OD_{600} of 0.005 in 20 mL of 10 mM phosphate buffer either in monoculture or in co-
276 culture. For the experiment Biolog EcoPlate™ were inoculated with 100 µl of each bacterial
277 inoculation suspension (monocultures or co-cultures) in each well. For each bacterial
278 monoculture one Biolog EcoPlate™ was inoculated, as well for each co-cultivation pair (*S.*

279 *plymuthica* PRI-2C with *H. gracilis* and *Paenibacillus* sp. AD87with *H. gracilis*). Plates were
280 incubated for 1 week and absorbance was measured at 590 nm every 24 hours on a BIOTEK
281 plate reader to determine the ability of the bacterial cultures to use the carbon sources present
282 in the wells.
283
284 **Trapping of volatile organic compounds and GC-Q-TOF analysis**
285 Please see Supplementary Methods.
286
287 **Ambient mass-spectrometry imaging LAESI-MS data analysis**
288 For LAESI-MS analysis a single colony of each bacterial isolate was picked and inoculated in
289 20 mL 1/10th TSB (*Paenibacillus* sp. AD87 and *S. plymuthica* PRI-2C) and grown overnight
290 at 24 °C at 220 rpm. For the inoculation of *H. gracilis* a single colony was picked from plate
291 and inoculated in 20 mL LB-medium and grown for three days at 24 °C at 200 rpm. The
292 inoculation mix was prepared by diluting the bacterial isolates in 20 mL of 10 mM Phosphate-
293 buffer (pH 6.5) to an OD_{600} of 0.005. The inoculum mix was pulse- vortexed for 30 sec. and a
294 droplet of 10 µl was added in the middle of a 6 cm diameter Petri dish (monocultures) or next
295 to each other in a distance of approx. 0.5 cm (pairwise interactions). All treatments were
296 inoculated in triplicates on 1/10th TSBA and incubated at 24 °C for five and ten days. After
297 five and ten days of incubation bacterial colonies were cut out of the agar (size approximately
298 1 – 3 cm^2) and subjected to LAESI-MS measurement. The LAESI-MS analysis was carried
299 out on a Protea Biosciences DP-1000 LAESI system (Protea Bioscience Inc., Morgantown,
300 WV, USA) coupled to a Waters model Synapt G2S (Waters Corporation, Milford, MA, USA)
301 mass spectrometer. The LAESI system was equipped with a 2940-nm mid-infrared laser
302 yielding a spot size of 100 µm. The laser was set to fire 10 times per x-y location (spot) at a
303 frequency of 10 Hz and 100% output energy. A syringe pump was delivering the solvent
304 mixture of methanol/water/formic-acid (50:50:0.1% v/v) at 2 µL/min to a PicoTip (5cm x 100
305 µm diameter) stainless steel nanospray emitter operating in positive ion mode at 4000 V. The
306 LAESI was operated using LAESI Desktop Software V2.0.1.3 (Protea Biosciences Inc.,
307 Morgantown, WV, USA). The Time of Flight (TOF) mass analyzer of the Synapt G2S was
308 operated in the V-reflectron mode at a mass resolution of 18.000 to 20.000. The source
309 temperature was 150 °C, and the sampling cone voltage was 30 V. The positive ions were
310 acquired in a mass range of 50 to 1200 *m/z*. The MS data was lock mass corrected post data
311 acquisition using leucine encephalin ($C_{25}H_{37}N_5O_7$ *m/z*= 556.2771), which was used as an
312 internal standard. All the acquired Waters *.RAW data files were converted to open file

313 format *.imzML using an in-house script written in R. Later, this data was pre-processed in
314 multiple steps to remove noise and to make the data comparable. First, square root
315 transformation was applied to the data to stabilize the variance. Then, baseline correction was
316 performed to enhance the contrast of peaks to the baseline. For better comparison of intensity
317 values and to remove small batch effects, Total-Ion-Current (TIC)-based normalization was
318 applied. This was followed by spectral alignment and peak detection to extract a list of
319 significant mass features for each sample replicate per treatment. In the end, a mass feature
320 matrix was generated with sample replicates for each treatment in columns and mass features
321 in rows. This feature matrix was used to perform further statistical analysis. The pre-
322 processing and peak-detection steps were applied using R scripts developed in-house and
323 using functions available within the MALDIquant R package (56). To perform multivariate
324 analysis, the feature matrix was imported into the online version of Metaboanalyst 4.0 (57).
325 Ion intensity maps displaying the spatial distribution for statistically significant mass features
326 were created using R. Before generating the ion maps, the intensity values for the selected
327 mass features were normalized to the maximum intensity within the image, measured for each
328 mass value individually. Venn diagrams displaying unique and common masses amongst
329 different treatments were drawn using the jvenn tool (58).
330

331 ## Results

332 **Isolation and identification of bacteria that pass through 0.1 µM filter**

333 Using a novel bacterial isolation and culture approach, we isolated bacteria from a terrestrial
334 soil sample that were able to pass through 0.22 µm and 0.1 µm pore-size filters. After several
335 days of incubation, only one type of bacterial colonies was observed on the inoculated plates.
336 The grown colonies were identified as *Hylemonella gracilis* (Gram-negative, class
337 betaproteobacteria, order Burkholderiales) by 16S rRNA sequence analysis.
338 The colonies showed a round and colorless morphology when grown on 1/10th TSBA plates
339 (**Fig. 1a**). Microscopically the bacteria had a spiraled morphology with a length of
340 approximately 6 - 12 µm, which is typical for *Hylemonella* species (**Fig. 1b**).
341

342 ***Hylemonella* grows better in interaction with other bacteria**

343 To test the hypothesis that small bacteria grow better in presence of normal-sized bacteria,
344 growth of *H. gracilis* was determined in co-culture with two phylogenetically distantly related
345 soil bacteria (*Paenibacillus* sp. AD87 and *S. plymuthica* PRI-2C) and compared to that of the
346 monoculture. The bacterial colony forming units of *H. gracilis* (CFU/mL) obtained on 1/10th

347 TSBA plates from monocultures and co-cultures are summarized in **Fig. 2**. Cell counts of
348 *Paenibacillus* sp. AD87 were 7.68 x 10^7 CFU/mL in co-culture with *H. gracilis* (**Fig. 2a**).
349 During the interaction with *H. gracilis*, the growth of *S. plymuthica* was significantly
350 negatively affected (*P=0.037*) after five days of incubation by reaching 1.47 x 10^9 CFU/mL
351 compared to the monocultures (**Fig. 2a**).
352 The bacterial colony forming units (CFU) obtained from *H. gracilis* grown in presence of cell
353 free supernatants (CFS) of *Paenibacillus* sp. AD87 and of *S. plymuthica* are summarized in
354 **Fig. 2b**. *H. gracilis* growth was significantly increased (*P*=0.011) when growing in presence
355 of cell free supernatants of *Paenibacillus* sp. AD87 resulting in higher *H. gracilis* cell counts
356 compared to the monoculture by reaching 1.10 x 10^6 CFU/mL. In the presence of cell free
357 supernatant from *S. plymuthica* PRI-2C*, H. gracilis* reached the highest cell counts at 1.72 x
358 10^6 CFU/mL (*P*=0.000) after five days of incubation (**Fig. 2b**).
359
360 **Interspecific interaction between bacterial species allows use of additional substrates**
361 During physiological or catabolic profiling, the metabolism of 31 carbon sources during
362 bacterial growth are measured in 96-wells plates. The catabolic profiling assays revealed that
363 *Paenibacillus* sp. AD87was able to utilize 11 out of the 31 carbon sources in monoculture,
364 while *S. plymuthica* PRI-2C and *H. gracilis* were able to utilize 17 and 16 carbon sources,
365 respectively. Interestingly, three compounds could be utilized only during the co-cultivation
366 of *H. gracilis* with one of the other species, these compounds could not be utilized by any of
367 the species in monoculture. Specifically, alpha- cyclodextrin was utilized only during the co-
368 cultivation of *H. gracilis* with *Paenibacillus* sp. AD87, while L-threonine and glycyl-L-
369 glutamic acid were utilized only during the co-cultivation of *S. plymuthica* PRI-2C and *H.*
370 *gracilis* (**Fig. 3).**
371
372 **Genomic features of *H. gracilis, S. plymuthica* PRI-2C *and Paenibacillus* sp. AD87**
373 Sequencing of the complete genome of *H. gracilis* resulted in a genome size of 3.82 Mbp with
374 3,648 coding sequences (CDS). As expected, the genome analysis revealed that the genome of
375 *H. gracilis* is smaller and contains fewer genes compared to *S. plymuthica* PRI-2C (5.4 Mbp)
376 and *Paenibacillus* sp. AD87 (7.0 Mbp). The genome features of all three bacteria are
377 summarized in **Table 1**.
378
379
380

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

382 The *in silico* tool *antiSMASH* allows the rapid genome-wide identification, annotation and
383 analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genomes
384 (59). *In silico* analysis of *Paenibacillus* sp. AD87 revealed a total of 10 gene clusters coding
385 for secondary metabolites. From which two gene clusters encode pathways for producing
386 terpenes, one for bacteriocins, one for lasso peptides, two for lanthipeptides, one for
387 nonribosomal peptides (NRPs), one for others, one for polyketides (type III enzyme
388 mechanism) and one gene cluster for non-NRP Siderophores (**Fig. 4a**). For *S. plymuthica*
389 PRI-2C, nine gene clusters, were found of which two gene clusters were annotated to encode
390 the production of NRPs, one of homoserine lactones, one of aryl polyenes and/or non-NRP
391 Siderophores, one of hybrid polyketide-NRP metabolites, one of thiopeptides, one of
392 butyrolactones, one of terpenes and one of others (**Fig. 4b**). For *H. gracilis* the AntiSMASH
393 analysis revealed that *H. gracilis* possesses relatively few gene clusters related to secondary
394 metabolism. A total of three gene clusters for *H. gracilis* were detected, of which one
395 belonged to the class of bacteriocins, one to the class of terpenes, and one to aryl polyenes,
396 the latter being a homolog to the aryl polyene gene cluster from *Xenorhabdus doucetiae*
397 (Genbank: NZ_FO704550.1) (**Fig. 4c)**

398

399 **Pathway analysis in *H. gracilis* compared to *S. plymuthica* PRI-2C and *Paenibacillus* sp.**
400 **AD87**

401 For annotation and Pathway analysis RAST (Rapid Annotation using Subsystem Technology)
402 and OrthoFinder were used. The RAST comparison of *Paenibacillus* sp. AD87 and *H.*
403 *gracilis* revealed 504 unique enzymes (according to their EC numbers) exclusive for
404 *Paenibacillus* sp. AD87, while 434 were present only in *H. gracilis* and 532 EC numbers
405 were shared by both genomes (**Fig. 5a**). The RAST comparison of *S. plymuthica* PRI-2C and
406 *H. gracilis* revealed that 751 enzymes were present only in *S. plymuthica* PRI-2C, and 260
407 were present only in *H. gracilis*. 727 EC numbers participating in diverse metabolic pathways
408 were found in both genomes (**Fig. 5b**).

409 The missing genes and pathways found by OrthoFinder and EggNOG were annotated with
410 GO terms. The analysis revealed that five genes related to metabolic pathways were absent in
411 *H. gracilis*. Those missing genes were annotated with the following molecular function
412 ontology terms: GO:0008473 (ornithine cyclodeaminase activity), GO:0008696 (4-amino-4-
413 deoxychorismatelyase activity), GO:0003920 (GMP reductase activity), GO:0004035
414 (alkaline phosphatase activity) and GO:0008442 (3-hydroxyisobutyrate dehydrogenase). We

415 verified if the absence of these molecular functions would render specific pathways obsolete
416 or unavailable in *H. gracilis*. However, alternative pathways routes are present for these genes
417 encoding certain molecular functions according to KEGG database annotations. The pathway
418 analysis by RAST did not reveal the absence of essential genes in *H. gracilis*. Still, the
419 comparison of the number (n) of genes present in each bacteria revealed major differences in
420 several pathways, specifically in the categories "Carbohydrates metabolism" and "Phosphorus
421 metabolism" (**Fig. 5b**). Interestingly, *H. gracilis* possesses no genes for those categories
422 according to RAST, whereas *Paenibacillus* sp. AD87possesses 393 and 82 genes, and *S.*
423 *plymuthica* PRI-2C 395 and 46 genes, respectively. A major difference in the absolute
424 number of genes in a category is also observed for Amino Acids and Derivatives, for which
425 *H. gracilis* possesses 318 genes, *Paenibacillus* sp. AD87 possesses 358 and *S. plymuthica*
426 PRI-2C 448 genes.
427
428 **Effect of interspecific interactions on gene expression**
429 The Transcriptome analysis of monocultures and co-cultures revealed a total of 277
430 significant differentially expressed genes; where from a total of 100 genes were down-
431 regulated and 177 genes were up-regulated between the different treatments (**Table 2**).
432
433 *Effect of inter-specific interactions on gene expression in Paenibacillus* sp. AD87 *and H.*
434 *gracilis*
435 Genes related signal transduction (T) were the category with the most differentially expressed
436 genes during the co-cultivation of *H. gracilis* with *Paenibacillus* sp. AD87 compared to the
437 monoculture of *H. gracilis* (**Supplementary Table 7** and **8**, **Fig. 6a, b**).
438 In *Paenibacillus* sp. AD87 histidine biosynthesis and dephosphorylation genes were up-
439 regulated (**Supplementary Table 2**), while cellular-growth-related genes were down-
440 regulated (**Supplementary Table 2**) at day 10 of the interaction with *H. gracilis* (**Fig. 6b**).
441 For the interaction of *H. gracilis* with *Paenibacillus* sp. AD87 15 significant differentially
442 expressed genes were found (0 at day five and 15 at day ten). At day five, genes related to
443 sulfur assimilation, chemotaxis and response to (chemical/external) stimuli were upregulated
444 in *H. gracilis* in the presence of *Paenibacillus* sp. AD87.
445
446 *Effect of inter-specific interactions on gene expression S. plymuthica* PRI-2C *and H. gracilis*
447 During the interaction of *S. plymuthica* PRI-2C with *H. gracilis*, 61 genes were significantly
448 differentially expressed at day five and 10 at day ten. At 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 3** and **4**). For *S. plymuthica* PRI-2C, 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 *S. plymuthica* PRI-2C, 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 organization/assembly and (iron)-sulfur cluster assembly were upregulated and genes related to the innate immune response (Toll Like Receptor signalling) were downregulated (**Supplementary Table 5** and **6**). At day ten, genes related to signal transduction and chemotaxis were upregulated in *H. gracilis*. For *H. gracilis*, the most upregulated genes were linked to chemotaxis pathway and iron scavenging, suggesting 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 3**). Of these, 17 were 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 a retention time (RT) of 26.4 min produced by the monocultures of *H. gracilis* was not detected in the interactions with *S. plymuthica* PRI-2C (**Table 3**). Two other unknown compounds with RT 4.15 min and with RT 24.34 min produced by the monocultures of *Paenibacillus* sp. AD87 were not detected in the co-cultivation with *H. gracilis* (**Table 3**).

483 **DART-MS based metabolomics**

484 Metabolomics analysis based on DART-MS revealed separations between the controls,
485 monocultures, and co-cultures as presented in PLS-DA score plots (**Fig. 7b**). The
486 metabolomic composition of the monocultures differed from that of the co-cultures (**Fig. 7b**).
487 Statistical analysis (ONE-WAY ANOVA and post-hoc TUKEY HSD-test) revealed 617
488 significant mass features present on day five and day ten of which 48 could be tentatively
489 assigned to specific compounds. Most of the significant peaks were found in the co-cultures
490 of *H. gracilis* with *Paenibacillus* sp. AD87. The significant mass features and the
491 corresponding tentative metabolites can be found in **Supplementary Table 10**.

492

493 **Mass spectrometry imaging metabolomics**

494 LAESI-MSI was performed to visualize the localization of metabolites in their native
495 environments in monoculture as well as during interaction without performing any extraction.
496 Across all treatments, clear separation was observed amongst the samples for controls,
497 monocultures and interactions **(Fig. 8a)**. An average of 1050 mass features was detected per
498 treatment. To list mass features that could explain separation amongst the controls,
499 monocultures and interactions, values of variable importance in projection (VIP) were
500 calculated. The top 40 statistically significant mass features with VIP scores > 2.0 are shown
501 in **Fig. 8b**. The box-and-whisker plots for the four statistically significant differentially
502 abundant metabolites selected from the volcano plot for the pair HM and PH are shown in
503 **Supplementary Figure 2a**. To visualize the statistically significant mass features between
504 monocultures and co-cultures samples in a pairwise manner, volcano plots were constructed
505 **(Supplementary Figure 3)**.

506 The volcano plot (**Supplementary Figure 3a**) for *H. gracilis* monoculture (HM) and the
507 interaction of *H. gracilis* with *Paenibacillus* sp. AD87 (PH) shows 53 mass features (in green)
508 located in the upper right quadrant, indicating that their concentrations are significantly higher
509 in HM as compared to PH. 18 mass features (in red) in the upper left quadrant of the plot have
510 a significantly lower concentration in HM as compared to PH. The ion intensity maps for
511 these statistically significant metabolites are shown alongside box-and-whisker plots. The ion
512 intensity maps are color coded based on the standard rainbow color scale where a pixel in red
513 represented a high concentration and the pixel in black represents no concentration of the
514 selected metabolite. As indicated, *m/z* 425.2886 and *m/z* 558.2832 show higher abundance in
515 interaction sample PH, whereas *m/z* 410.8587 and *m/z* 716.7610 display high abundance in
516 HM as compared to PH. For the pairwise analysis performed for *Paenibacillus* sp. AD87

517 monoculture (PM) and the co-culture of *H. gracilis* with *Paenibacillus* sp. AD87 (PH), 149
518 mass features (in green) displayed significantly high concentration in PM and 75 mass
519 features (in red) had significantly low concentration in PM as compared to PH
520 (**Supplementary Figure 3b**). This is also evident in the box-and-whisker plots and the ion
521 intensity maps that are presented for four statistically significant metabolites belonging to this
522 set (**Supplementary Figure 2b)**.
523 For the pairwise analysis for *H. gracilis* monoculture (HM) and the co-culture of *S.*
524 *plymuthica* PRI-2C and *H. gracilis* (SH), 57 mass features (in green) displayed significantly
525 high concentration in HM and 42 mass features had significantly low concentration in HM as
526 compared to SH (**Supplementary Figure 3c**). The box-and-whisker plots along with the ion
527 intensity maps for four statistically significant metabolites belonging to this set are shown in
528 **Supplementary Figure 2c**. For the pairwise analysis for *S. plymuthica* PRI-2C monoculture
529 (SM) and the interaction of *S. plymuthica* PRI-2C and *H. gracilis* (SH), 135 mass features (in
530 green) displayed significantly high concentration in SM and 65 mass features had
531 significantly low concentration in SM as compared to SH (**Supplementary Figure 3d**). The
532 box-and-whisker plots along with the ion intensity maps for four statistically significant
533 metabolites belonging to this set are shown in **Supplementary Figure 2d**.
534 To visualize the number of shared and unique metabolites amongst the monoculture and
535 interaction samples Venn diagrams were plotted. The Venn diagram (**Supplementary Figure**
536 **3e**) for monocultures *H. gracilis* and *Paenibacillus* sp. AD87 and their interaction shows 80
537 metabolites unique to *H. gracilis* monoculture, 75 metabolites unique to *Paenibacillus* sp.
538 AD87 monoculture and 100 metabolites that are unique during their interaction. 1062
539 metabolites were shared within these three treatments. Similarly, the Venn diagram
540 **(Supplementary Figure 3f)** for monocultures *H. gracilis* and *S. plymuthica* PRI-2C and their
541 interaction shows 196 metabolites unique to *H. gracilis* monoculture, 48 metabolites unique
542 to *S. plymuthica* PRI-2C monoculture and 120 metabolites that are unique during their
543 interaction.
544

545 ## Discussion
546
547 Here we report the first time isolation of *H. gracilis* from a terrestrial soil sample. This
548 bacterium passed a 0.1 μm filter, which suggests a very small cell size, theoretically justifying
549 referring to these bacteria as ultra-small bacteria (26). However, against our expectation, the
550 microscopical analysis revealed that this bacterium is not ultra-small in cell size but possesses
551 a very thin diameter and showed the typical spiraled morphology known for these species (60-

552 63). These observations are in line with previous research by Wang *et al*. showing that *H.*
553 *gracilis* is capable of passing through filters of various pore sizes ranging from 0.45 μM to
554 0.1μM (64), most probably thanks to their cell shape and cell morphology. *In silico* analysis
555 of 16 terrestrial metagenome data available on MG-RAST (https://www.mg-rast.org/) showed
556 that *H. gracilis* was not present in terrestrial metagenome data (not shown) suggesting that H.
557 gracilis is not commonly present in terrestrial soils. The bacterial interaction assays revealed
558 that *H. gracilis* grows faster when interacting with *Paenibacillus* sp. AD87 or *S. plymuthica*
559 PRI-2C. The cell numbers of *H. gracilis* were higher when exposed to cell-free supernatants
560 of *Paenibacillus* sp. AD87 and *S. plymuthica* PRI-2C, suggesting that the metabolites
561 released by the latter bacteria in co-cultures with *H. gracilis* are associated with improved
562 growth of *H. gracilis*. We hypothesized that *H. gracilis* grows better in co-culture, either
563 because growth is stimulated by signals produced by the other organism, or because the
564 environment that is created by the other organism allows *H. gracilis* to make more efficient
565 use of certain metabolic pathways. Indeed, the metabolic experiments with BioLog™ plates
566 showed that during interspecific interactions of *H. gracilis* with *Paenibacillus* sp. AD87 or
567 with *S. plymuthica* PRI-2C, more carbohydrates could be utilized compared to the
568 monocultures. This is an interesting observation, and it may indicate that interaction of
569 bacteria can trigger the production of exo-enzymes enabling the degradation of carbohydrates,
570 which the bacteria were not able to degrade in monoculture.
571 We speculated that since *H. gracilis* grows better in interaction with other bacteria and is of
572 relatively small cell size, *H. gracilis* might have evolved according to a genome streamlining
573 strategy, i.e. the adaptive loss of genes for which functions it relies on interaction with other
574 bacteria in the immediate environment. The whole-genome sequencing of *H. gracilis* revealed
575 a genome size of 3.82 Mbp. This is a relatively small genome size for free-living soil bacteria
576 that typically have estimated average genome sizes of ~4.7 Mbp (34, 65-68). The *in silico*
577 antiSMASH (43) comparison of genes that are part of secondary metabolite gene clusters
578 showed that the *H. gracilis* genome contained only three gene clusters encoding the
579 production of secondary metabolites (bacteriocins, terpenes, and aryl polyenes). Terpenes and
580 aryl polyenes are known as protective compounds against abiotic stressors, while bacteriocins
581 have antimicrobial activities against closely related bacteria (17, 69-73). We hypothesize that
582 *H. gracilis* genome streamlining has allowed it to be more competitive, by retaining only the
583 most essential metabolic functions while having roughly about one quarter less DNA to
584 replicate during each cell division. Gene loss and reduced genome size may cause dependency
585 on other microbes in their surroundings, and this may explain a considerable part of the

586 phenomenon that most of the detectable bacteria in the environment are not cultivable under
587 laboratory conditions.

588 To investigate the mechanisms of interaction, we performed transcriptome analysis on
589 the interaction pairs of *H. gracilis* with *S. plymuthica* PRI-2C and *Paenibacillus* sp. AD87.
590 Interestingly, a higher amount of significantly differentially expressed genes was induced by
591 *H. gracilis* in the other two competing bacteria as compared to the transcriptomic changes in
592 *H. gracilis.* Several processes, enriched according to GO term enrichment analysis, could be
593 part of a mechanism(s) mediating interactions between *H. gracilis* and *S. plymuthica* PRI-2C
594 and *Paenibacillus* sp. AD87*,* for example genes related to chemotaxis. Moreover, the GO
595 terms for signal transduction, secondary metabolite production and, cell motility were
596 enriched in the transcriptome of *H. gracilis* during the co-cultivation with *Paenibacillus* sp.
597 AD87, suggesting that chemotaxis and cell movement is an important feature during
598 interspecific interactions between these two bacterial taxa (74, 75). In addition, GO terms
599 referring to Iron-sulfur (Fe-S) complex assembly were enriched in the transcriptomes of *H.*
600 *gracilis* during the co-cultivation with *S. plymuthica* PRI-2C and *Paenibacillus* sp. AD87. Fe-
601 S clusters are important for sustaining fundamental life processes: they participate in electron
602 transfer, substrate binding/activation, iron or sulfur storage, regulation of gene expression,
603 and enzyme activity (76, 77). This up-regulation could indicate that, potentially, in co-culture,
604 normal-sized bacteria released metabolites that *H. gracilis* used for synthesizing Fe-S
605 complexes. It is also possible that iron-sulfur complex assembly is activated during
606 competition with the interacting bacteria for sulfur, or iron collection (scavenging) (78-81).

607 The metabolic pathway analysis showed that the loss of genes in *H. gracilis* does not
608 appear to have resulted in functional loss of metabolic pathways. Loss of non-essential and
609 possibly redundant genes in several metabolic pathways could explain why and how the
610 genome of *H. gracilis* has become so small. The missing genes are not essential to complete
611 metabolic pathways and only appear to result in limited options in certain metabolic
612 pathways. RAST analysis showed that all basal metabolic pathways remain feasible with the
613 annotated enzymes and pathways of *H. gracilis*. The only exception is EC term 5.2.1.1
614 (maleate isomerase) (it would help to specify in which pathway this enzyme is reported);
615 There are several ways to synthesize fumarate, e.g. in the glycolysis pathway (63, 82, 83) and
616 in the citric acid cycle (63, 84). Based on the available data, it cannot be unambiguously
617 determined which alternative pathway may preferably be used by *H. gracilis* to synthesize
618 fumarate.

619 The metabolomics analysis revealed the production of specific antimicrobial
620 compounds such as pyrollnitrin (*S. plymuthica* PRI-2C) and 2,5-bis(1-methylethyl)-pyrazine
621 (*Paenibacillus* sp. AD87) which are well known for their broad-spectrum antimicrobial
622 activity (85-89). However, the produced antimicrobial compounds didn't show activity
623 against *H. gracilis*: in both interactions, *H. gracilis* showed increased growth when growing
624 in co-culture with either *Paenibacillus* sp.AD87 or *S. plymuthica* PRI-2C.
625 The understanding of natural metabolites that mediate interactions between organisms
626 in natural environments is the key to elucidate ecosystem functioning. The detection and
627 identification of the compounds that mediate such interactions is still challenging. Techniques
628 such as mass spectrometry imaging (MSI) provide new opportunities to study
629 environmentally relevant metabolites in their spatial context (90-92). In this study, the
630 metabolomics was performed using three independent approaches namely DART-MS
631 analysis, GC/MS-Q-TOF analysis and Laser Ablation Electrospray Ionization-Mass
632 Spectrometry Imaging mass spectrometry (LAESI- IMS) from living bacterial colonies.
633 LAESI Imaging MS analysis revealed that several mass features were detected in higher
634 abundance during the co-cultivation of *H. gracilis* with *Paenibacillus* sp. AD87, these mass
635 features were m/z 425.2886 and m/z 558.2832. LAESI-MSI is not suitable for unambiguous
636 compound annotation, but LAESI- MSI can still be used for putative compound annotation.
637 To annotate the detected mass features to compounds with high certainty, LAESI mass
638 spectrometry imaging should be coupled with ion mobility separation as suggested by (93-
639 95). Yet, LAESI-MSI can help to spatially distinguish the produced secondary metabolites of
640 living bacterial colonies with limited sample preparation and can give insight into the spatial
641 distribution of metabolites.
642 Several studies indicate that the volatile blend composition of the volatiles greatly depends on
643 biotic interactions and on growth conditions (15, 19, 96-98). Here, a higher number of volatile
644 compounds were detected in the bacterial co-cultures, most likely due to the combination of
645 emitted volatiles of the interacting bacteria. The high numbernumber of sulfur-containing
646 compounds indicates that these compounds are commonly produced by bacteria and might
647 play an important role in signaling during interspecific interactions (99, 100). No novel
648 volatile compounds were detected during the co-culture of the three bacteria.
649 Overall, our study showed that *H. gracilis* is able to pass through 0.1 µM filter, and is present
650 in terrestrial environments. The growth performance and physiological behavior of *H. gracilis*
651 were dependent on the co-cultivated bacterial partner and they might be metabolically
652 depending on the co-cultivated bacteria. At the same time, *H. gracilis* was able to change the

physiology, release of volatile organic compounds and secreted enzymes of the co-cultivated bacteria without direct cell-cell contact.

Microbial interspecific interactions play an important role in the functioning of the terrestrial ecosystem. Soil microbial communities are very diverse and dynamic and involve frequent and sporadic interspecific interactions. Our study indicates that sulfur and Fe-S clusters could play important role in microbial interspecific interactions in terrestrial environments and more studies are required to understand their role.. The study of sporadic interspecific interactions and the inclusion of rare taxa in future analysis could help to better understand microbial communities and functions of those. Could you exemplify how this study improved our understanding? production of sulfur compounds and Fe-S clusters maybe?

Data availability

The raw data of this article will be made available by the authors to any qualified researcher upon request. The whole genome sequence of *Hylemonella gracilis* 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 # PRJNA483535.

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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. All authors read and critically revised the manuscript.

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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Figures and Tables

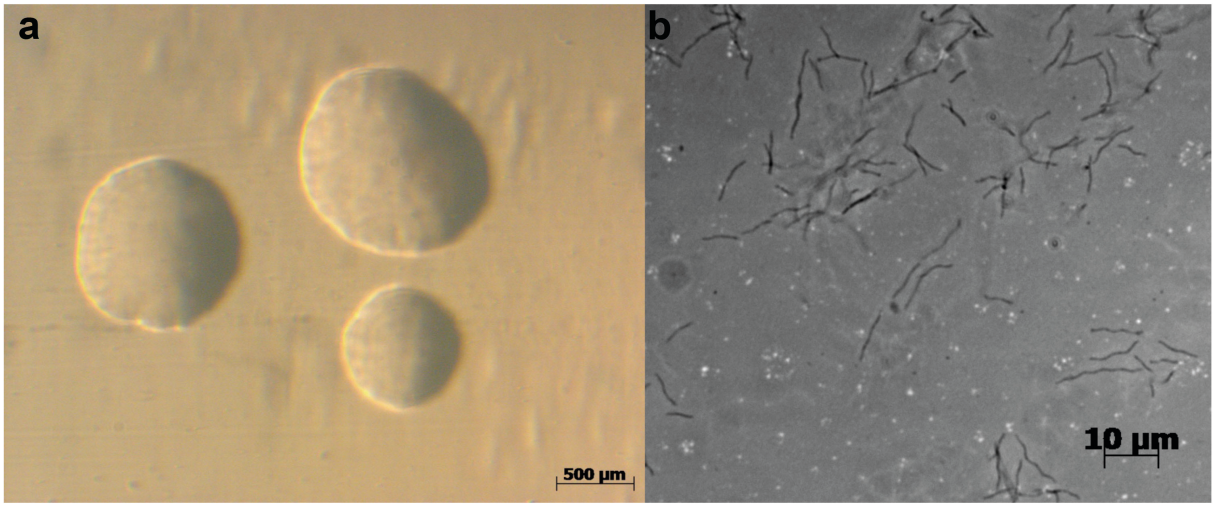


Figure 1: Morphology of *Hylemonella gracilis* (**a**) on 1/10th TSB- agar plates captured at 20x magnification and (**b**) single bacterial cells captured at 400 X magnification showing their very thin, long and slender appearance in liquid media.

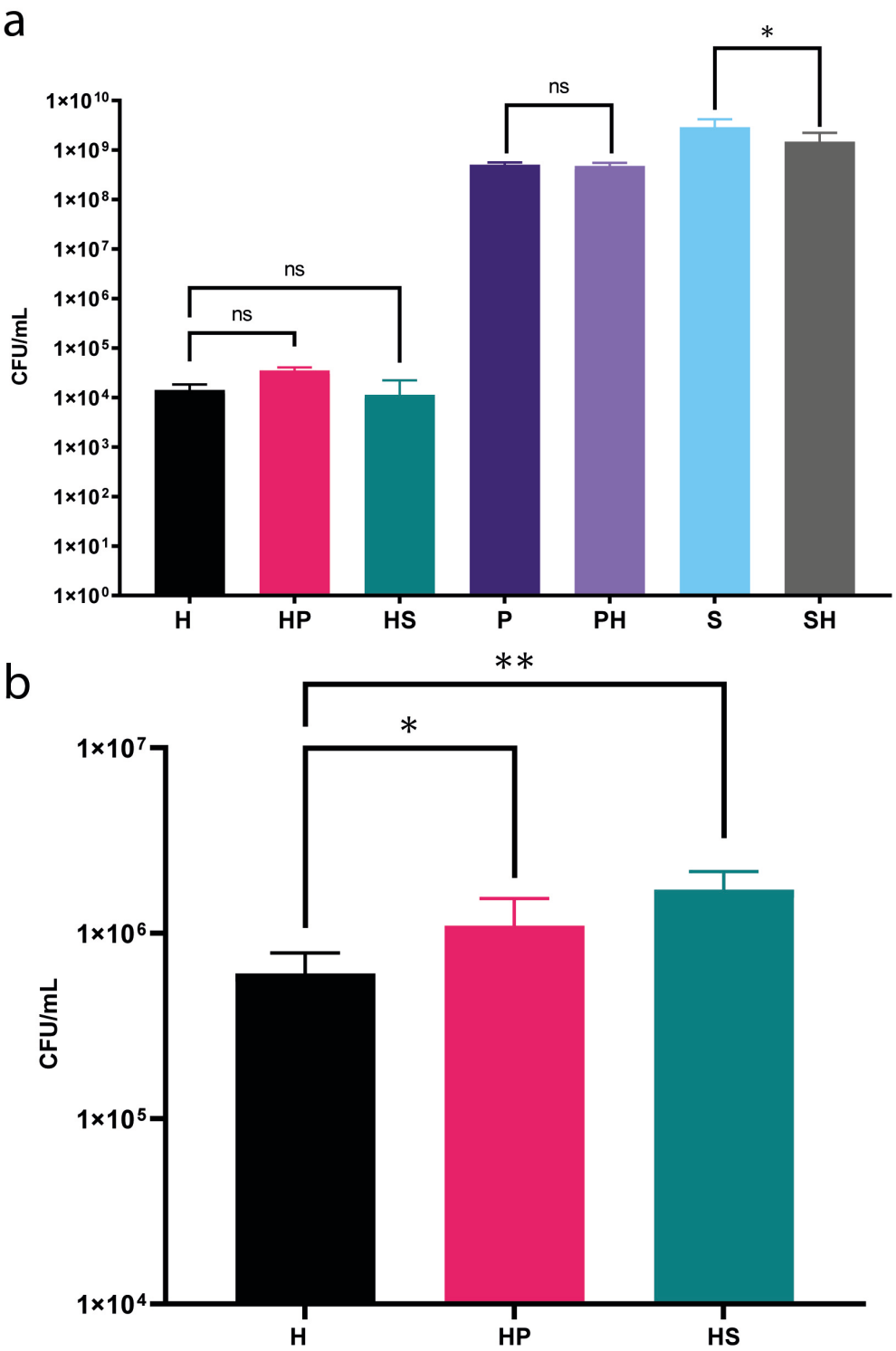
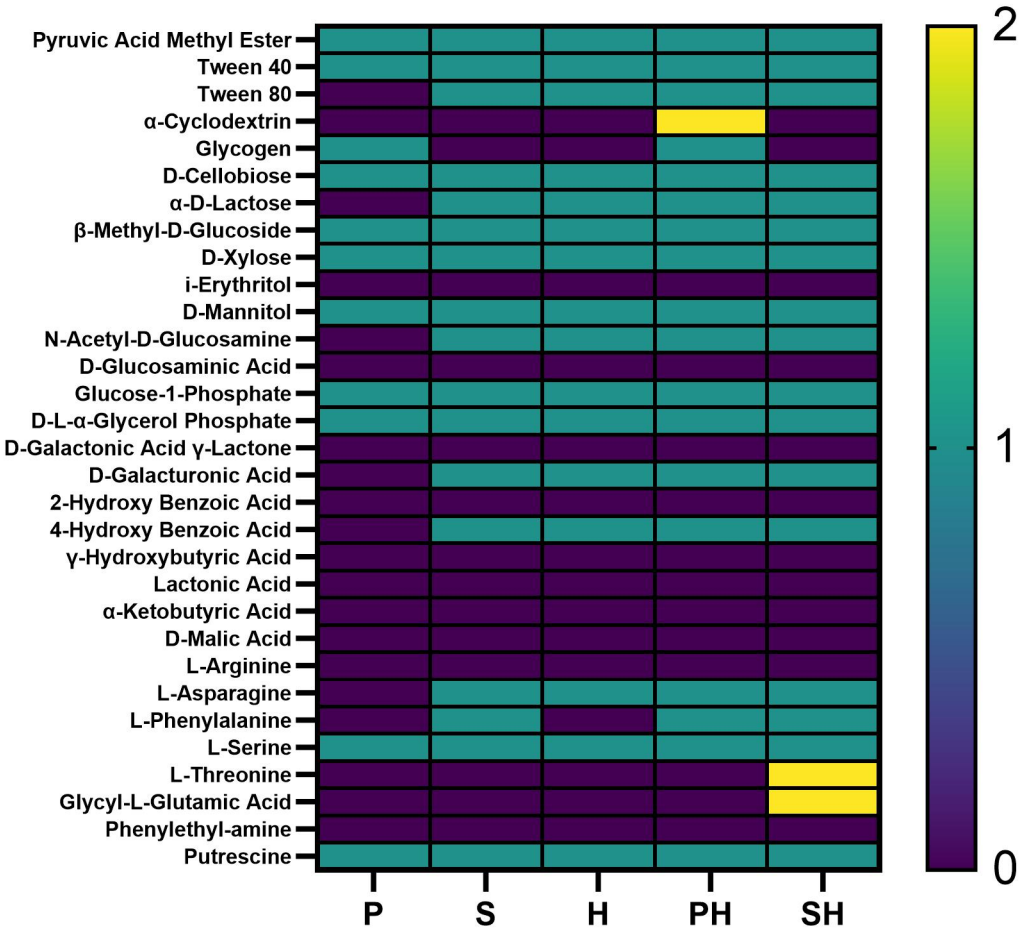


Figure 2: Growth of bacterial mono and cocultures (a) on the plate-based experiment and (b) during the cell-free-supernatant (CFS) experiment. Abbreviations: *H. gracilis* monoculture (H), *Paenibacillus* sp. AD87 monoculture (P), *Paenibacillus* sp. AD87 – *H. gracilis* coculture (HP), *S. plymuthica* PRI-2C monoculture (S), *S. plymuthica* - *H. gracilis* coculture (SH). *H. gracilis* - *Paenibacillus* sp. AD87 coculture (HP), *H. gracilis* – *S. plymuthica* PRI-2C coculture (HS). Significant differences in colony forming units per

1004 milliliter (CFU/mL) between co-cultures (treatment) and monocultures (controls) are
1005 indicated by asterisks (ONE-WAY ANOVA, post-hoc TUKEY test).



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1007 **Figure 3: Results of the Biolog EcoPlate™ experiment.** Bacteria were inoculated in
1008 monoculture or in pairwise combinations on the EcoPlate™ with 31 different carbon sources.
1009 Colour code: turquoise=carbon source could be utilized in monoculture (1), yellow= carbon
1010 source could be utilized only in co-culture (2) purple= carbon source could not be utilized (0).
1011 Abbreviations: *Paenibacillus* sp. AD87 monoculture (P), *Paenibacillus* sp. AD87 - *H. gracilis*
1012 coculture, (PH) *H. gracilis* monoculture (H), *S. plymuthica* PRI-2C monoculture (S), *S.*
1013 *plymuthica* PRI-2C - *H. gracilis* coculture (SH).

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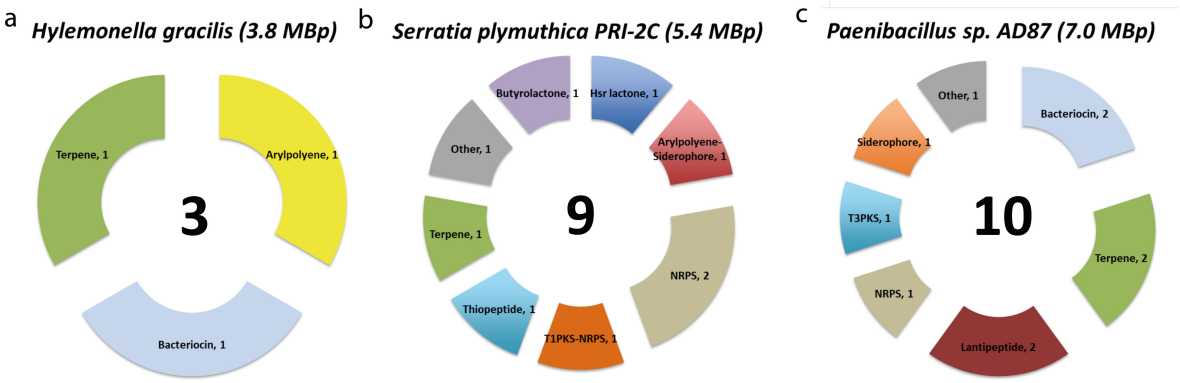

Figure 4: *In silico* comparison of biosynthetic gene clusters (BGCs) predicted by antiSMASH in the genomes of three soil bacteria. From left to right (**a**) *H. gracilis* with a genome size of 3.8 MBp, n= 3 gene clusters for secondary metabolites, (**b**) *S. plymuthica* PRI-2C with a genome size of 5.4 MBp, n=9 gene clusters for secondary metabolites and (**c**) *Paenibacillus* sp. AD87 with a genome size of 7.0 MBp, n=10 gene clusters for secondary metabolites.

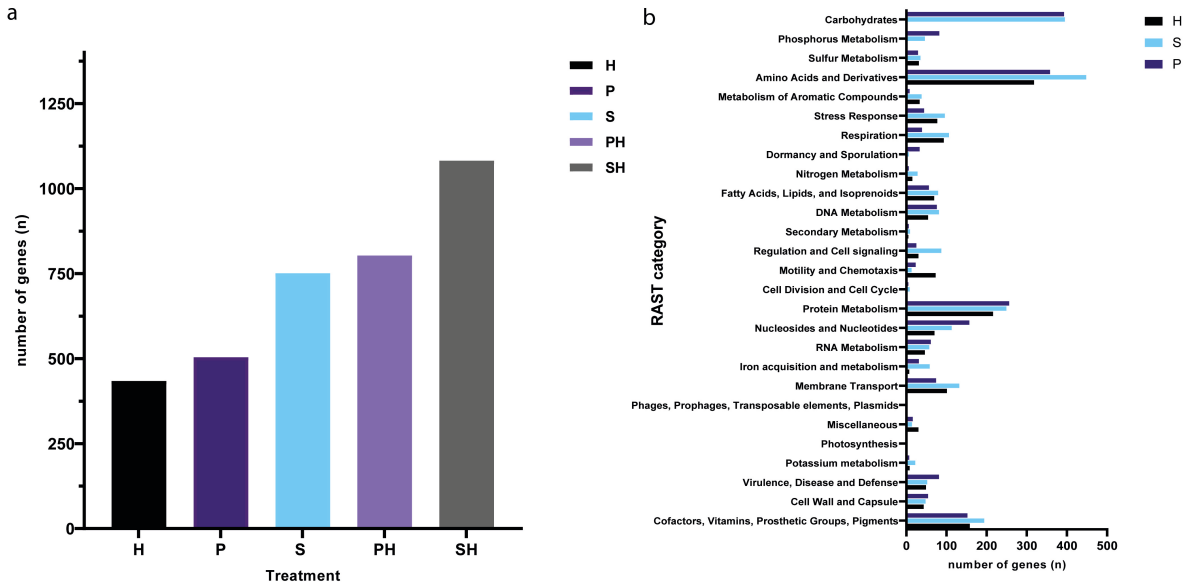

Figure 5: Gene content comparison. (**a**) Box-plot showing the number (n) of all expressed genes (n) for the monocultures of *H. gracilis* (H), *Paenibacillus* sp. AD87 monoculture (P) and *S. plymuthica* PRI-2C monoculture (S) and during the interaction of *H. gracilis* with *Paenibacillus* sp. AD87 (PH) and for the interaction of *H. gracilis* with *S. plymuthica* PRI-2C (SH) determined by RAST. (**b**) Boxplot showing number (n) of expressed genes present in each RAST subsystem category for each of the monocultures.

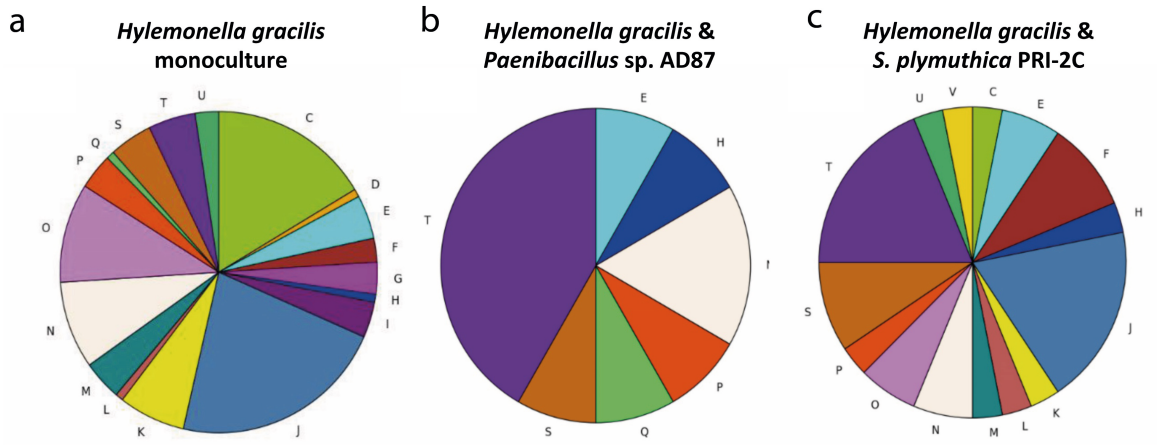

Figure 6: Pie-charts representing up-regulated genes identified by differential gene expression analysis and COG annotation (**a**) *Hylemonella gracilis* monoculture gene expression level (**b**) *H. gracilis* in co-culture with *Paenibacillus* sp. AD87; (**c**) *H. gracilis* co-culture with *S. plymuthica* 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. **COG- Abbreviations**: C: energy production and conversion; D: cell cycle control, cell division, chromosome partitioning; E: amino acid transport and metabolism; F: nucleotide transport and metabolism; G: carbohydrate transport and metabolism; H: coenzyme transport and metabolism; I: lipid transport and metabolism; J: translation, ribosomal structure and biogenesis; K: transcription; L: replication, recombination and repair; M: cell wall/membrane/envelope biogenesis; N: cell motility; NA: not assigned; O: posttranslational modification, protein turnover; chaperones; P: inorganic ion transport and metabolism; Q: secondary metabolites biosynthesis, transport and catabolism; R: general function prediction only; S: function unknown; T: signal transduction mechanisms; U: intracellular trafficking, secretion, and vesicular transport; V: defense mechanisms.

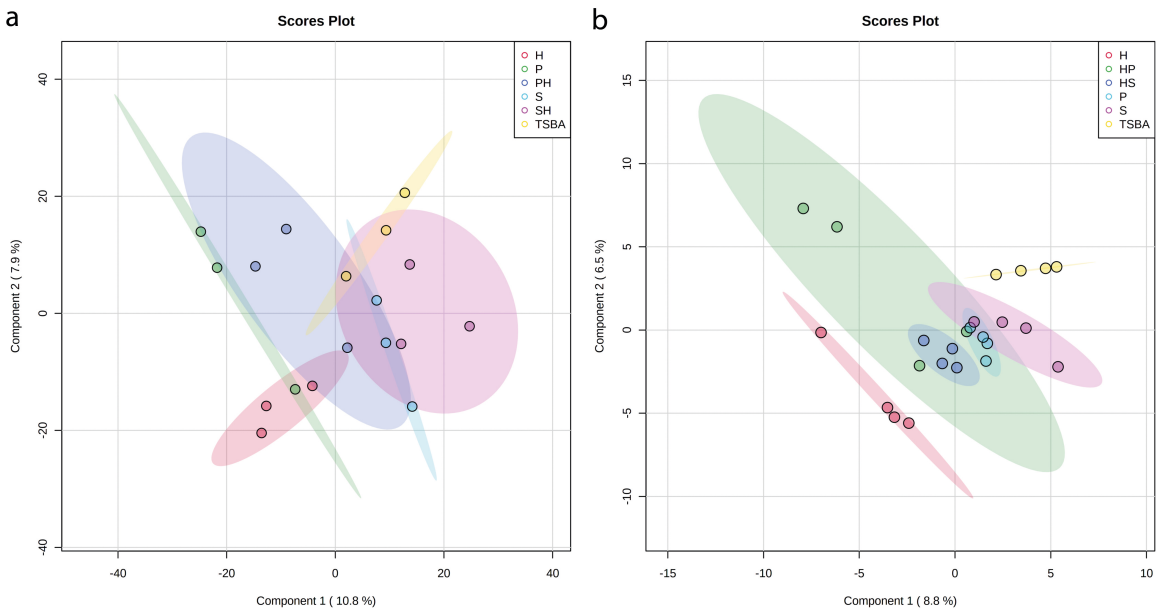

Figure 7: PLS-DA plots of the metabolomics data (**a**) PLS-DA 2D- plots of volatiles emitted by monocultures and pairwise combinations of *H. gracilis*, *Paenibacillus* sp. AD87 and *Serratia plymuthica* after ten days of inoculation, time point (t=10 days) (**b**) PLS-DA 2D-plots of DART-MS data of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

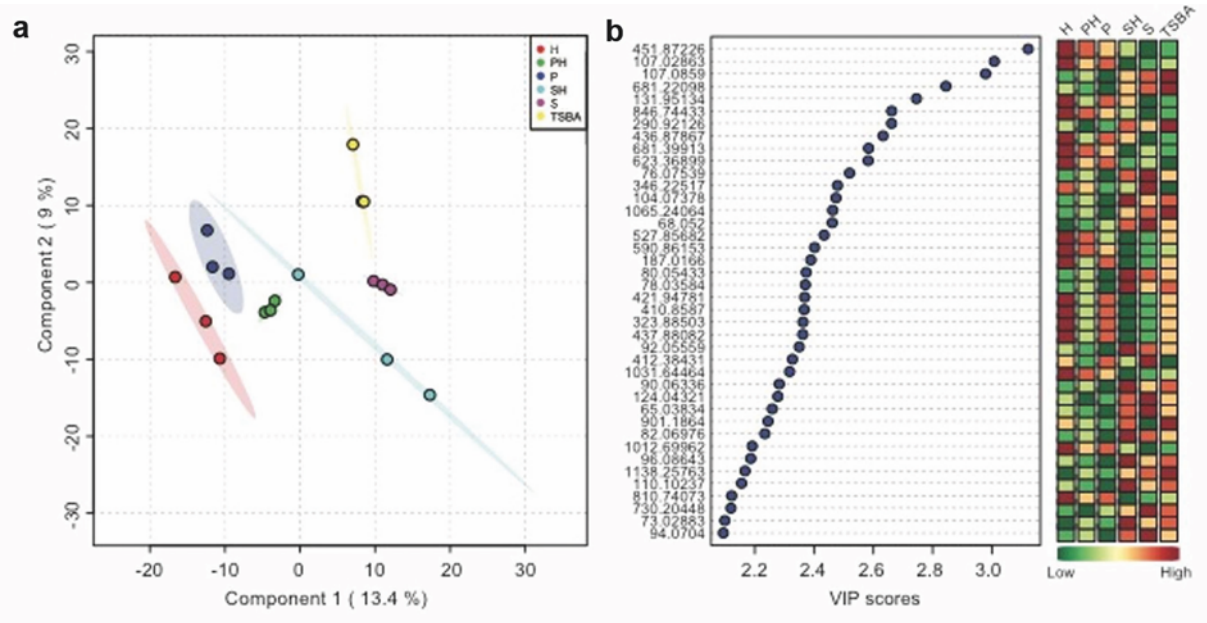

Figure 8: PLS-DA plots of the first 40 significant mass features observed in LAESI-MSI data. (a) PLS-DA score plot for *H. gracilis* monoculture (H), *Paenibacillus* sp. AD87 monoculture (P), *Paenibacillus* sp. AD87 – *H. gracilis* co-culture (PH), *S. plymuthica* PRI-2C monoculture (S), *S. plymuthica* PRI-2C - *H. gracilis* co-culture (SH) and TSBA control (TSBA). **(b)** Top 40 statistically significant features identified by PLS-DA based on Variable

1077 Importance in Projection (VIP) score. The colored boxes on the right indicate the relative
1078 concentrations of the corresponding metabolite in each group under study.

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1080 # Tables

1081 **Table 1**: Genome assembly statistics and outcome of *in silico* analysis of secondary
1082 metabolite gene clusters of *H. gracilis, S. 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**

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1088 **Table 2**: Overview of the transcriptome analysis, number (n) of significantly differentially
1089 expressed genes of *H. gracilis* responding to *S. plymuthica* PRI-2C or to *Paenibacillus* sp.
1090 AD87, *Serratia plymuthica* PRI-2C responding to *H. gracilis* and *Paenibacillus* sp. AD87
1091 responding to *H. gracilis* at day 5 and day 10.

Organism	Interacting organism	time point (d)	Significantly differentially expressed genes		Total (n)
			up-regulated	down-regulated	
Serratia plymuthica PRI-2C	*Hylemonella gracilis*	5	25	36	61
		10	10	0	10
Paenibacillus sp. AD87		5	8	0	8
		10	0	0	0
Hylemonella gracilis	*Serratia plymuthica* PRI-2C	5	1	0	1
		10	129	53	182
	Paenibacillus sp. AD87	5	0	0	0
		10	4	11	15
Total (n)			**177**	**100**	**277**

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1099 **Table 3:** Tentatively identified volatile organic compounds (VOCs) produced by a *H.*
1100 *gracilis, S. plymuthica* PRI-2C and *Paenibacillus* sp. AD87 strains in mono- and cocultures.

						Detected in bacterial culture				
#	Compound name	RT*	ELRI**	p-value***	chemical class	H	S	P	SH	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**

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1102 Abbreviations:
1103 *H. gracilis* monoculture, SM= *Serratia plymuthica* PRI-2C monoculture, PM= *Paenibacillus*
1104 sp. AD87 monoculture, PH= *Paenibacillus* sp. AD87 and *H. gracilis* co-culture, SH= *Serratia*
1105 *plymuthica* PRI-2C and *H. gracilis* co-culture
1106 # = Compound number
1107 RT* = Retention time, the RT value stated is the average of three technical replicates.
1108 ELRI** = Experimental linear retention index value, the RI value stated is the average of
1109 three replicates.
1110 p-value***= statistical significance (peak area and peak intensity)
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