

1 **The intestinal microbiota predisposes to traveller's diarrhoea and to the carriage of multidrug-**
2 **resistant Enterobacteriaceae after travelling to tropical regions.**

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38 **Abstract**

39 The risk of acquisition of multidrug-resistant Enterobacteriaceae (MRE) and of occurrence of
40 diarrhoea is high when travelling to tropical regions. The relationships between these phenomena and
41 the composition of human gut microbiota have not yet been assessed. Here, we investigated the
42 dynamics of changes of metabolically active microbiota by sequencing total RNA from faecal samples
43 taken before and after travel to tropical regions. We found that the occurrence of diarrhoea during the
44 travel was associated with a higher relative abundance of *Prevotella copri* before departure and after
45 return. The composition of microbiota, before travel as well as at return, was not correlated with the
46 acquisition of MRE. However, the clearance of MRE one month after return was linked to a specific
47 pattern of bacterial species that was also found before and after return.

48

49 **Introduction**

50

51 Multidrug-resistant Enterobacteriaceae (MRE) that produce extended-spectrum beta-lactamases
52 (ESBLs), plasmid-encoded AmpC-type cephalosporinases (pAmpC) and/or carbapenemases (CP)
53 have been spreading in the community over the last two decades [1]. MRE represent a major public
54 health issue, as a limited number of antibiotics remains active against these bacteria while very
55 innovative antibiotics are expected to reach the market in the near future [2]. The spread of MRE has
56 been particularly intense in tropical regions, likely owing to poor hygiene conditions and uncontrolled
57 antibiotic usage [3]. Consequently, between 14% and 69% of travellers to tropical regions are
58 reported to acquire MRE, depending on the cohort and specific destination [4, 5]. Besides, a high
59 proportion of travellers to these destinations also report the occurrence of diarrhoea (traveller's
60 diarrhoea) during their trip [4-8]. Among travellers who acquire a MRE during a travel to tropical
61 regions, the observed median carriage is short (≤ 1 month) [5, 6]. Still, some of them experience long-
62 term carriage of MRE, extending up to 1 year in 2.2-11% cases [5, 6].

63 The capacity of the intestinal microbiota to resist long-term settlement of exogenous bacteria
64 (including MRE) is referred to as colonization resistance [9-11] which is mainly exerted by anaerobes
65 [9, 10]. Antibiotics with high activity against anaerobes strongly affect the capacity of the microbiota to
66 prevent colonization by exogenous microorganisms, and thus favour the acquisition and expansion of
67 resistant bacteria [12]. Hence, the restoration of the intestinal microbiota through faecal material
68 transplantation (FMT) has been shown to lower the intestinal concentrations of vancomycin-resistant

69 enterococci (VRE) [13], MRE [14] and more globally, of antibiotic resistance genes [15]. Moreover, the
70 administration of a limited set of intestinal bacteria to mice colonised with VRE reduced the intestinal
71 densities of VRE, suggesting that specific bacteria are involved in colonization resistance [16].

72 To date, the link between the composition of the intestinal microbiota and the acquisition of MRE
73 during travel and their clearance after return has not been assessed.

74 Here, we questioned whether the composition of the intestinal microbiota could be associated with the
75 occurrence of diarrhoea and the acquisition of MRE during travel and to the clearance of MRE after
76 return.

77

78 **Methods**

79 *Population*

80 The travellers cohort originates from the VOYAG-R study (ClinicalTrials.gov n°NCT01526187) funded
81 by the French Ministry of Health [6, 17]. From February 2012 to April 2013, subjects attending six
82 international vaccination centres in the Paris area, prior to traveling to tropical regions, were asked to
83 provide faecal samples before and after their trip. Only volunteers who had no detectable MRE in the
84 faeces taken before their departure, were asked to send a further stool sample within a week after
85 their return. Travellers who revealed positive for MRE after their return were asked to provide
86 additional stool samples 1, 2, 3, 6 and 12 months after their return until MRE was no longer detected.
87 Among the 574 included subjects, 292 (51%) acquired at least one MRE [6]. For the present analysis,
88 a specific sub-cohort of 43 subjects (15 from Sub-Saharan Africa, 13 from Latin America and 15 from
89 Asia) were chosen. No particular criteria of inclusion were applied except the fact that we wanted to
90 have almost the same number of travellers per tropical region. In addition to a native stool sample,
91 volunteers were asked to ship a stool aliquot diluted in another 200 mL vial containing 50 mL of
92 RNeasy® solution (Applied Biosystems, Villebon-sur-Yvette, France) for metagenomic analysis. In
93 this sub-cohort of 43 travellers, 18 subjects (41.8%) acquired an MRE (mostly ESBL-producing
94 *Escherichia coli*) during their trip. All of them provided a stool sample one month after they returned,
95 among whom 6 were still carrying a MRE. Thus, 104 samples were selected (43 before travel, 43 at
96 return and 18 one month after travel).

97

98

99 *RNA extraction from stool samples*

100 Total RNA (mostly made of ribosomal RNA e.g. 16S, 23S and 5S) was extracted for 104 stool
101 samples using the RNeasy Plus Mini Kit (Qiagen, Gaithersburg, USA). The concentration of RNA
102 obtained was measured by Qubit RNA BR Assay Kit or Qubit RNA HS Assay Kit (Life Technologies,
103 Reinach, Switzerland). For simplicity, the terms 16S and 23S refer to 16S and 23S ribosomal RNAs,
104 respectively. The integrity of RNA (ratio 16S/23S) was assessed by the RNA 6000 Nano Kit and RNA
105 6000 Pico Kit (Agilent Technologies, Plan-les-Ouates, Switzerland) on the Bioanalyzer 2100 system
106 (Agilent Technologies, Waldbronn, Germany). The detailed protocol can be found in the
107 Supplementary Methods.

108

109 *Sequencing and reads processing*

110 One hundred ng of purified RNA in 10 μ L total volume were sent to LGC (Berlin, Germany) for
111 (i) cDNA synthesis using NEBNext mRNA First/Second Strand Synthesis Module (New England
112 Biolabs, Ipswich, USA) (ii) shotgun library preparation using NuGEN Ovation Rapid Library System
113 (NuGEN, San Carlos, USA), and (iii) sequencing (2 x 300bp) of size-selected fragments (about 300-
114 400 bp) using v3 Illumina chemistry and half the capacity of a MiSeq (Illumina, San Diego, USA) flow
115 cell. The paired reads were merged using BBMerge 35.43 (<http://bbmap.sourceforge.net>), trimmed
116 and quality-filtered by Mothur v1.35.1 [18]. The average number of merged quality-filtered pairs
117 exploitable for mapping was 89,351 (median = 87,728) per sample, the average length of reads after
118 merging was 355 (range = 200–560). Quality-filtered merged reads were mapped onto the 16S
119 Greengenes operational taxonomic units (OTUs) database pre-clustered at 97% identity [19] with
120 USEARCH [20] by considering an identity of $\geq 97\%$ and $\geq 97\%$ query coverage (see Supplementary
121 Methods). Downstream analyses were carried out using the taxonomic information of the matching
122 full-length reference 16S gene sequences. In parallel, we aligned quality-filtered merged reads
123 against the SILVA ribosomal 23S/28S database [21] with USEARCH using the same settings as for
124 the 16S profiling. Then, we selected the reads that mapped to bacterial 23S genes but had no hit
125 against eukaryotic 28S sequences. Reads classified to the same taxon were summed up. The
126 mapping rates to 16S and 23S genes are reported in Supplementary Methods. The relative
127 abundance of a given OTU or taxon was computed as percentage of the total number of reads in a
128 sample, mapping to that OTU or taxon.

129 *Bio-statistical analyses*

130 The variables used in this study are detailed in the Supplementary Methods. To assess similarities
131 and differences between bacterial communities in response to predictor variables, we performed
132 principal coordinates analysis (PCoA) either in PRIMER v6 (PRIMER-E Ltd, Plymouth, UK) or in the R
133 software v3.2.3 with the vegan v2.3-5 and ade4 v1.7-4 packages. Permutational analysis of variance
134 (global and pairwise PERMANOVA, with 9,999 permutations, unrestricted permutation of raw data
135 and Type III sums of squares) and distance-based linear models (DistLM, with 9999 permutations)
136 based on the Bray-Curtis similarity matrices of square-root transformed OTUs' or taxa relative
137 abundance were carried out in PRIMER. Ecological indices were determined after rarefying the 16S
138 data set to the lowest number of reads assigned to the OTUs in any of the samples (10,465).
139 Observed richness (S) and Shannon diversity were calculated in PRIMER.

140 Taxa with significantly different relative abundances between two conditions were identified in R
141 software using the Wilcoxon rank sum test or Wilcoxon signed rank test (with a significance threshold
142 of $p < 0.05$ without adjustments for multiple comparisons [22]). Besides, OTUs contributing to
143 discrimination between groups of samples were also identified using similarity percentage analysis
144 (SIMPER, PRIMER-E) and the on-line tool Linear discriminant analysis Effect Size (LeFSe) [23].

145

146 *De novo 16S gene assembly*

147 Samples taken before departure from 39 subjects who were not treated with antibiotics during the
148 travel were considered for this analysis. First, reads mapping to a given OTU were collected from
149 each sample and assembled with CAP3 (version date: 02/10/15) [24] using default settings. Then, the
150 16S genes assembled from each sample and corresponding to the same OTU were aligned and the
151 consensus sequence was determined again with CAP3. Finally, the taxonomic assignment of the
152 obtained consensus sequence of 16S genes was done by BLASTing [25] against the non-redundant
153 16S SILVA and Greengenes databases using a minimum percentage identity of 97. Hits with the
154 highest percentage of identity and lowest e-value of alignment were retained. In case of multiple
155 choices with equal scores, the hit with the lowest taxonomic rank was selected.

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160 **Results**

161 *Effect of travelling on microbiota composition*

162 To test the hypothesis whether the modalities and the duration of travelling to tropical regions has an
163 impact on microbiota composition, we analysed the microbiota profiles at return of 39 travellers that
164 were not administered antibiotics (except doxycycline for malaria prophylaxis) during their trip. We did
165 not observe any significant influence on microbiota composition in relation to the visited region, the
166 type of travel, duration of travel, to the use and the type of malaria prophylaxis (Table 1). However,
167 the proportions of Enterobacteriaceae increased during the travel in all the 39 individuals
168 (Supplementary Figure 5).

169 Moreover, we analysed microbiota profiles before departure, at return and one month after the return
170 of 17 travellers who acquired MRE during their journey and were not treated with antibiotics. We
171 observed that the faecal samples taken at different time points (before departure, at return and one
172 month after return) clustered by subject (global PERMANOVA test, p-value <0.0001; Supplementary
173 Figure 12), while travel did not have a significant impact on microbiota profiles (pairwise
174 PERMANOVA tests, p-values ranged between 0.3-0.9).

175

176 *Occurrence of diarrhoea during the travel*

177 In pre-travel microbiota of people who suffered from diarrhoea during the travel (n=9) the relative
178 abundance of *Prevotella copri* species was >2-fold higher than in subjects who did not experience this
179 condition (n=34) (Wilcoxon rank sum test, p-value <0.05; Supplementary Figure 1C-D). Nevertheless,
180 the overall composition of pre-travel microbiota was not significantly associated with the occurrence of
181 diarrhoea during travel (Table 2; Supplementary Figure 1A).

182 We, then, analysed the intestinal microbiota profiles at return by excluding those 4 travellers who had
183 taken antibiotics during their trip. Microbiota profiles from subjects who had diarrhoea significantly
184 differed from those who had not (Tables 2; Figure 1A). The occurrence of diarrhoea was associated
185 with a microbiota significantly less rich and diverse at return as compared to the microbiota of
186 individuals who did not experience this condition during the travel (Wilcoxon rank sum test, p-value
187 <0.05; Figure 1B).

188 At return, people having experienced diarrhoea during their travel presented increased proportions of
189 Bacteroidetes and Proteobacteria phyla and decreased proportions of Firmicutes phylum compared to
190 travellers without diarrhoea (Figure 1C). The great majority of OTUs found to be differentially
191 abundant after travel between people with and without diarrhoea mapped to Enterobacteriaceae
192 family, Clostridiales order and the *P. copri* species (LEfSe and Wilcoxon rank sum test, p-value <
193 0.05; Figure 1D; Supplementary Figures 2A, 5, 6, 7). In particular, the faecal samples taken at return
194 from travellers who reported diarrhoea showed a >2-fold higher proportion of Enterobacteriaceae and
195 *Prevotella copri* and a <2-fold lower proportion of Clostridiales as compared to travellers without
196 diarrhoea. The relative abundance of *P. copri* was increased at return only in a fraction of travellers
197 reporting diarrhoea (Supplementary Figure 6).

198 The results obtained for the samples collected at return from travel were also confirmed by 23S
199 analyses (Supplementary Figure 2B-C-D-E). Most Enterobacteriaceae members associated with
200 diarrhoea mapped to 23S genes of *Escherichia-Shigella*, while in the 16S analysis the discriminating
201 OTUs from Enterobacteriaceae were not classified at the genus level. Clostridiales included genera
202 belonging to Ruminococcaceae (*Ruminococcus*), Bacteroidaceae (*Bacteroides*) and Lachnospiraceae
203 (*Roseburia*; only for 16S dataset: *Lachnospira* and *Blautia*).

204 We then investigated whether diarrhoea had protracted effects on gut bacteria composition one
205 month after the return. Therefore, we analysed samples from 17 travellers who did not take antibiotics
206 during the first month following the return. We did not detect significant differences in the overall
207 microbiota composition in relation to diarrhoea (Table 2, Supplementary Figure 8A).
208 Enterobacteriaceae relative abundance returned close to basal level in all 17 travellers but with
209 statistical significance only when the analyses included the individuals who did not suffer from
210 diarrhoea (Wilcoxon signed rank test, p-value <0.05, Supplementary Figure 9). One month after
211 return, *Prevotella copri* abundance was still increased in travellers who had diarrhoea (Supplementary
212 Figure 6) whereas Clostridiales members were more abundant in individuals without diarrhoea
213 (Supplementary Figure 7).

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215
216

Acquisition and carriage of MRE

217 The acquisition of MRE was not associated with a specific microbiota profile neither before travel nor
218 at return (Table 2; Supplementary Figures 1B, 10).

219 We also investigated the association of microbiota composition and the MRE carriage one month after
220 return in the 17 travellers found MRE-positive at return and not treated with antibiotics. In this case,
221 the composition of the intestinal microbiota of the subjects whose samples became MRE-negative
222 (n=11) was significantly different from those whose samples remained MRE-positive (n=6) (Table 2).
223 OTUs assigned to *Bifidobacterium adolescentis* (OTU6129) and to some Clostridiales (OTU61,
224 OTU7384, OTU405, OTU8089) as well as *Bifidobacterium* strains detected by 23S analyses, were
225 enriched in individuals who had cleared MRE one month after the return (LEfSe and Wilcoxon rank
226 sum test, p-value <0.05; Figure 2A-B; Supplementary Figure 11A-B). Already before departure, the
227 proportions of these bacteria were higher in MRE carriers who cleared their carriage one month after
228 return than in those who were still positive (Figure 2A; Supplementary Figure 11A). For most OTUs
229 associated with MRE clearance, the species level taxonomy of *de novo* assembled 16S gene was not
230 available (Supplementary Table 1).

231 Remarkably, within the subcohorts of 17 travellers, we found that the microbiota profiles of MRE
232 cleared individuals were significantly different from persistent MRE carriers before departure and one
233 month after return (Supplementary Table 2; Figure 2C; Supplementary Figure 11C).

234 The observed species richness measured before travel was significantly lower in individuals who
235 acquired MRE during the travel and remained MRE carriers one month after return than in those who
236 cleared their carriage (Supplementary Figure 8B). We found that the relative abundance of several
237 OTUs and strains/species mapping to Enterobacteriaceae significantly decreased more than 2 fold
238 one month after the return, independently of MRE carriage (Supplementary Figures 13). Despite that,
239 the abundance of Proteobacteria phylum, to which Enterobacteriaceae belong, did not change over
240 time or in relation to MRE carriage measured one month after the return (Figure 2D; Supplementary
241 Figure 11D).

242

243 **Discussion**

244

245 One of the main results of this study is that travellers who experienced diarrhoea (regardless of the
246 aetiology) had higher intestinal abundance of *P. copri*, before the travel, at return, and one month
247 after, than those who did not. This suggests that subjects with higher relative abundance of *P. copri*
248 could be at higher risk for diarrhoea during travel. Interestingly, *P. copri* has popped up in various

249 metagenomic studies as either a beneficial or pathogenic bacterium: it has been associated to
250 rheumatoid arthritis [26, 27], insulin resistance [28] and parasitic diarrhoea in children [29], but also to
251 good health status [30, 31] and to an improved glucose homeostasis [32]. These contradictory
252 findings could be explained by the high inter-strain and inter-individual genetic variations of this
253 species [33], suggesting that different strains have various functions, antigens and/or metabolites
254 being either beneficial or deleterious for the host. Another characteristic of microbiota profiles of
255 people suffering diarrhoea is decreased species richness at return. Diminution of species richness
256 upon diarrhoea occurrence is also described in one study conducting by 16S profiling the intestinal
257 microbiota of travellers returning to USA from Central America or India [34]. Oppositely, subjects
258 suffering from a Norovirus-caused diarrhoea had a surprisingly higher intestinal richness and diversity
259 than diarrhoea-free travellers. However, in this study low numbers of reads per sample (<3000) were
260 analysed and pre-travel samples were not assessed [34].

261 We observed that the travellers who did not experience diarrhoea during travel had a microbiota
262 profile enriched in members of *Ruminococcus*, *Coprococcus*, and *Dorea*. Consistently with our
263 findings, these genera were depleted in 16S microbiota profiles of individuals suffering from post-
264 transplantation [35] and nosocomial diarrhoea, including *Clostridium difficile* infection [36]. Moreover,
265 *Roseburia* and *Ruminococcus* species have been shown to prevent gut inflammation by
266 strengthening gut barrier function in mice [37] and by enhancing starch fermentation in humans [38],
267 respectively. Subjects who did not experience diarrhoea during the travel had a richer and more
268 diverse microbiota after travel than before. However, one month after return, the diversity and
269 richness for those individuals tended to decrease to a baseline (pre-travel) level. Increase in richness
270 and diversity at return when diarrhoea is not experience could reflect the ingestion of new bacteria
271 that are not met in France, and/or the consumption of food that could act as prebiotics for bacteria in
272 the pre-travel sample.

273 Besides, we observed an increase of Enterobacteriaceae in all travellers, which was more significant
274 in those subjects who experienced diarrhoea during their trip. This could be expected as several
275 diarrhoea agents - *E. coli*, *Shigella* and *Salmonella* - belong to the Enterobacteriaceae family [39].
276 This increase was transient and the relative abundance of Enterobacteriaceae returned close to
277 baseline one month after travel.

278 Another significant result of this study is the association between intestinal microbiota composition
279 and clearance of MRE in healthy travellers. Indeed, we observed that a set of bacteria from the
280 Clostridiales order was more abundant in travellers who had no detectable MRE one month after
281 return than in those who remained MRE carriers. Moreover, this pattern was already observed before
282 travel. In addition, among the subjects who acquired MRE during travel, bacterial richness and
283 diversity at baseline (before travel) were lower in individuals who remained MRE carriers one month
284 after travel than in those who did not. Altogether, our findings support the concept that the intestinal
285 microbiota before travel may not predispose to the acquisition of MRE, but will affect its clearance in
286 case of acquisition. These results are in line with the observations performed in mice in which some
287 specific OTUs were shown to be associated with the clearance of vancomycin-resistant enterococci
288 [16] - *Listeria monocytogenes* [40] and *Clostridium difficile* [41] - but also with the MRE clearance after
289 faecal material transplantation [14]. Nonetheless, these phenomena were observed after an alteration
290 of the microbiota by antibiotics, whereas our observations were obtained from healthy, antibiotic-free
291 travellers.

292 The main limitation of this study is that the number of samples is relatively low with regards to some
293 variables such as MRE carriage after the return and the occurrence of diarrhoea. Nonetheless, we
294 combined several bio-statistical and bioinformatics approaches which produced consistent results and
295 therefore strengthen our findings. Of note, we took advantage of using total RNA (rich in ribosomal
296 RNA) to bypass the need for amplification of a specific region of the 16S gene (that leads to biases)
297 and to allow a separate analysis on 16S and 23S taxonomic markers. Hence, despite the low number
298 of samples in some sub-groups, we are confident with our conclusions because of the consistency of
299 the results throughout the various bio-statistical and bioinformatic analyses performed. Another
300 limitation is that we could not precisely identify the bacterial species associated with the intestinal
301 clearance of MRE despite our attempts to assemble the full 16S genes, hindering the realization of in
302 vitro and in vivo experiments to demonstrate the precise role of these bacteria. Metagenomic
303 sequencing that allows functional analysis could now be used on a similar setting to identify the genes
304 associated to the clearance of MRE irrespectively of the taxonomy of their host. Finally, the
305 aetiological agents of traveller's diarrhoea were not sought as it was outside of the VOYAG-R study's
306 scope. Consequently, we could not link the composition of the microbiota to the presence of a specific

307 pathogen. Still, the intestinal alteration due to traveller's diarrhoea seems to be pathogen-independent
308 [34].

309 In conclusion, we showed that the composition of intestinal microbiota is associated with the risk of
310 occurrence of diarrhoea in healthy travellers and of carriage of MRE in those who acquired MRE
311 during the travel. Our results call for further functional explorations of the interplay between the
312 intestinal microbiota, traveller's diarrhoea and MRE carriage.

313

314 **Data Availability**

315 Mothur-quality-filtered sequences were deposited as fastq files at the European Nucleotide Archive
316 (ENA) under the project PRJEB24843. Prior to sequences' submission, reads assigned to human
317 genome (vGRCh38.p10) by CLARK (v1.2.3.2) [42] were removed. CLARK taxonomic classification
318 was performed at the phylum level (Chordata).

319

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326

327 **Author contributions**

328 ER, LAL and AA conceived and designed the study. ER, JS and VL supervised the study. The
329 VOYAG-R study group performed the princeps's study. MG and SL performed RNA extraction. E.R.,
330 V.L., S.L. designed the statistical analyses. SL and ER analysed the data. NG helped with read pre-
331 processing. SL, VL and ER wrote the manuscript. SL assembled all the figures. CE and SM revised
332 the manuscript. All authors read and approved the final manuscript.

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341

342 **Competing financial interests.**

343 All authors have no potential conflicts of interest.

344 **References**

- 345 1. Pitout, J.D.D. and K.B. Laupland, *Extended-spectrum beta-lactamase-producing*
346 *Enterobacteriaceae: an emerging public-health concern*. The Lancet. Infectious Diseases,
347 2008. **8**: p. 159-166.
- 348 2. Boucher, H.W., et al., *10 x '20 Progress--development of new drugs active against gram-*
349 *negative bacilli: an update from the Infectious Diseases Society of America*. Clinical Infectious
350 Diseases, 2013. **56**: p. 1685-1694.
- 351 3. Woerther, P.-L., et al., *Trends in human fecal carriage of extended-spectrum β -lactamases in*
352 *the community: toward the globalization of CTX-M*. Clinical Microbiology Reviews, 2013. **26**:
353 p. 744-758.
- 354 4. Tängdén, T., et al., *Foreign travel is a major risk factor for colonization with Escherichia coli*
355 *producing CTX-M-type extended-spectrum beta-lactamases: a prospective study with*
356 *Swedish volunteers*. Antimicrobial agents and chemotherapy, 2010. **54**: p. 3564-3568.
- 357 5. Arcilla, M.S., et al., *Import and spread of extended-spectrum β -lactamase-producing*
358 *Enterobacteriaceae by international travellers (COMBAT study): a prospective, multicentre*
359 *cohort study*. The Lancet Infectious Diseases, 2017. **17**(1): p. 78-85.
- 360 6. Ruppé, E., et al., *High Rate of Acquisition but Short Duration of Carriage of Multidrug-*
361 *Resistant Enterobacteriaceae After Travel to the Tropics*. Clinical Infectious Diseases: An
362 Official Publication of the Infectious Diseases Society of America, 2015. **61**: p. 593-600.
- 363 7. Kantele, A., et al., *Antimicrobials increase travelers' risk of colonization by extended-spectrum*
364 *betalactamase-producing Enterobacteriaceae*. Clinical Infectious Diseases: An Official
365 Publication of the Infectious Diseases Society of America, 2015. **60**: p. 837-846.
- 366 8. Steffen, R., D.R. Hill, and H.L. DuPont, *Traveler's diarrhea: A clinical review*. JAMA, 2015.
367 **313**(1): p. 71-80.
- 368 9. Ducluzeau, R., J.C. Salomon, and J. Huppert, *[Implantation of a lysogenic strain and a*
369 *sensitive strain of Escherichia coli K 12 in the digestive tube of axenic mice: establishment of*
370 *a balance]*. Annales De l'Institut Pasteur, 1967. **112**: p. 153-161.
- 371 10. Vollaard, E.J. and H.A. Clasener, *Colonization resistance*. Antimicrobial Agents and
372 Chemotherapy, 1994. **38**: p. 409-414.
- 373 11. Buffie, C.G. and E.G. Pamer, *Microbiota-mediated colonization resistance against intestinal*
374 *pathogens*. Nature Reviews. Immunology, 2013. **13**: p. 790-801.
- 375 12. van der Waaij, D., J.M. Berghuis-de Vries, and n. Lekkerkerk Lekkerkerk-v, *Colonization*
376 *resistance of the digestive tract in conventional and antibiotic-treated mice*. The Journal of
377 Hygiene, 1971. **69**: p. 405-411.
- 378 13. Stripling, J., et al., *Loss of Vancomycin-Resistant Enterococcus Fecal Dominance in an*
379 *Organ Transplant Patient With Clostridium difficile Colitis After Fecal Microbiota Transplant*.
380 Open Forum Infectious Diseases, 2015. **2**: p. ofv078.
- 381 14. Singh, R., et al., *Donor feces infusion for eradication of Extended Spectrum beta-Lactamase*
382 *producing Escherichia coli in a patient with end stage renal disease*. Clinical Microbiology and
383 Infection: The Official Publication of the European Society of Clinical Microbiology and
384 Infectious Diseases, 2014.
- 385 15. Millan, B., et al., *Fecal microbial transplants reduce antibiotic-resistant genes in patients with*
386 *recurrent Clostridium difficile infection*. Clinical Infectious Diseases: An Official Publication of
387 the Infectious Diseases Society of America, 2016.
- 388 16. Ubeda, C., et al., *Intestinal microbiota containing Barnesiella species cures vancomycin-*
389 *resistant Enterococcus faecium colonization*. Infection and Immunity, 2013. **81**: p. 965-973.
- 390 17. Ruppé, E., et al., *Acquisition of carbapenemase-producing Enterobacteriaceae by healthy*
391 *travellers to India, France, February 2012 to March 2013*. Euro Surveillance: Bulletin
392 Européen Sur Les Maladies Transmissibles = European Communicable Disease Bulletin,
393 2014. **19**.
- 394 18. Schloss, P.D., et al., *Introducing mothur: open-source, platform-independent, community-*
395 *supported software for describing and comparing microbial communities*. Appl Environ
396 Microbiol, 2009. **75**.
- 397 19. DeSantis, T.Z., et al., *Greengenes, a chimera-checked 16S rRNA gene database and*
398 *workbench compatible with ARB*. Applied and Environmental Microbiology, 2006. **72**: p. 5069-
399 5072.
- 400 20. Edgar, R.C., *Search and clustering orders of magnitude faster than BLAST*. Bioinformatics,
401 2010. **26**(19): p. 2460-2461.

- 402 21. Quast, C., et al., *The SILVA ribosomal RNA gene database project: improved data processing*
403 *and web-based tools*. Nucleic Acids Research, 2013. **41**: p. D590-D596.
- 404 22. Benjamini, Y. and Y. Hochberg, *Controlling the False Discovery Rate: A Practical and*
405 *Powerful Approach to Multiple Testing*. Journal of the Royal Statistical Society. Series B
406 (Methodological), 1995. **57**: p. 289-300.
- 407 23. Segata, N., et al., *Metagenomic biomarker discovery and explanation*. Genome Biology,
408 2011. **12**: p. R60.
- 409 24. Huang, X. and A. Madan, *CAP3: A DNA sequence assembly program*. Genome Research,
410 1999. **9**: p. 868-877.
- 411 25. Altschul, S.F., et al., *Basic local alignment search tool*. Journal of Molecular Biology, 1990.
412 **215**: p. 403-410.
- 413 26. Maeda, Y., et al., *Dysbiosis Contributes to Arthritis Development via Activation of*
414 *Autoreactive T Cells in the Intestine*. Arthritis & Rheumatology (Hoboken, N.J.), 2016. **68**: p.
415 2646-2661.
- 416 27. Pianta, A., et al., *Evidence for Immune Relevance of Prevotella copri, a Gut Microbe, in*
417 *Patients with Rheumatoid Arthritis*. Arthritis & Rheumatology (Hoboken, N.J.), 2016.
- 418 28. Pedersen, H.K., et al., *Human gut microbes impact host serum metabolome and insulin*
419 *sensitivity*. Nature, 2016. **535**: p. 376-381.
- 420 29. Gilchrist, C.A., et al., *Role of the Gut Microbiota of Children in Diarrhea Due to the Protozoan*
421 *Parasite Entamoeba histolytica*. The Journal of Infectious Diseases, 2016. **213**: p. 1579-1585.
- 422 30. Glick-Bauer, M. and M.-C. Yeh, *The health advantage of a vegan diet: exploring the gut*
423 *microbiota connection*. Nutrients, 2014. **6**: p. 4822-4838.
- 424 31. Kovatcheva-Datchary, P., et al., *Dietary Fiber-Induced Improvement in Glucose Metabolism Is*
425 *Associated with Increased Abundance of Prevotella*. Cell Metabolism, 2015. **22**: p. 971-982.
- 426 32. De Vadder, F., et al., *Microbiota-Produced Succinate Improves Glucose Homeostasis via*
427 *Intestinal Gluconeogenesis*. Cell Metabolism, 2016. **24**: p. 151-157.
- 428 33. Zhu, A., et al., *Inter-individual differences in the gene content of human gut bacterial species*.
429 Genome Biology, 2015. **16**: p. 82.
- 430 34. Youmans, B.P., et al., *Characterization of the human gut microbiome during travelers'*
431 *diarrhea*. Gut Microbes, 2015. **6**(2): p. 110-119.
- 432 35. Lee, J.R., et al., *Gut Microbial Community Structure and Complications Following Kidney*
433 *Transplantation: A Pilot Study*. Transplantation, 2014. **98**(7): p. 697-705.
- 434 36. Antharam, V.C., et al., *Intestinal Dysbiosis and Depletion of Butyrogenic Bacteria in*
435 *Clostridium difficile Infection and Nosocomial Diarrhea*. Journal of Clinical Microbiology, 2013.
436 **51**(9): p. 2884-2892.
- 437 37. Patterson, A.M., et al., *Human Gut Symbiont Roseburia hominis Promotes and Regulates*
438 *Innate Immunity*. Frontiers in Immunology, 2017. **8**: p. 1166.
- 439 38. Ze, X., et al., *Ruminococcus bromii is a keystone species for the degradation of resistant*
440 *starch in the human colon*. The ISME Journal, 2012. **6**(8): p. 1535-1543.
- 441 39. Shah, N., H.L. DuPont, and D.J. Ramsey, *Global etiology of travelers' diarrhea: systematic*
442 *review from 1973 to the present*. Am J Trop Med Hyg., 2009.
- 443 40. Becattini, S., et al., *Commensal microbes provide first line defense against Listeria*
444 *monocytogenes infection*. The Journal of Experimental Medicine, 2017. **214**: p. 1973-1989.
- 445 41. Buffie, C.G., et al., *Precision microbiome reconstitution restores bile acid mediated resistance*
446 *to Clostridium difficile*. Nature, 2014.
- 447 42. Ounit, R., et al., *CLARK: fast and accurate classification of metagenomic and genomic*
448 *sequences using discriminative k-mers*. BMC Genomics, 2015. **16**(1): p. 236.

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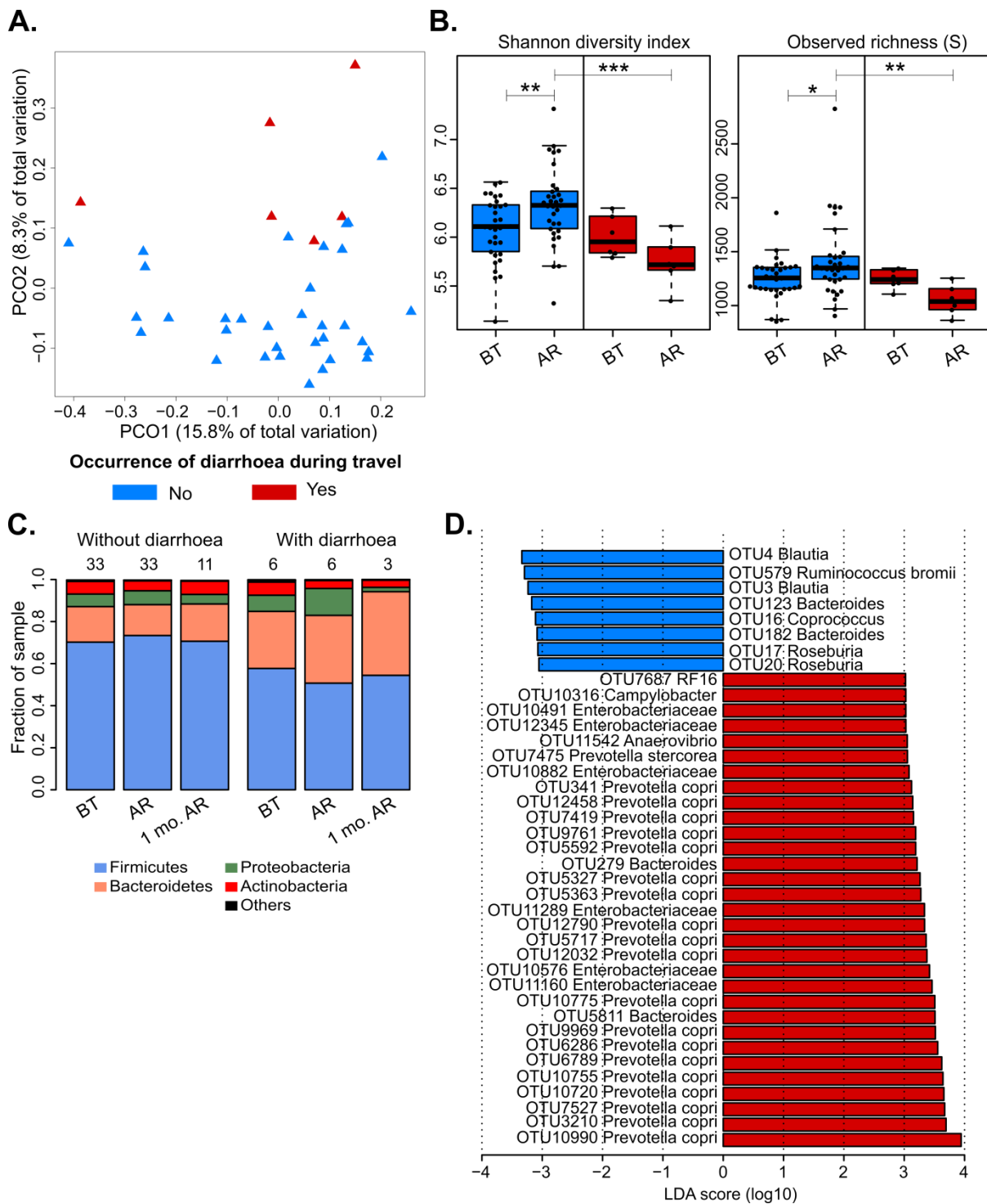
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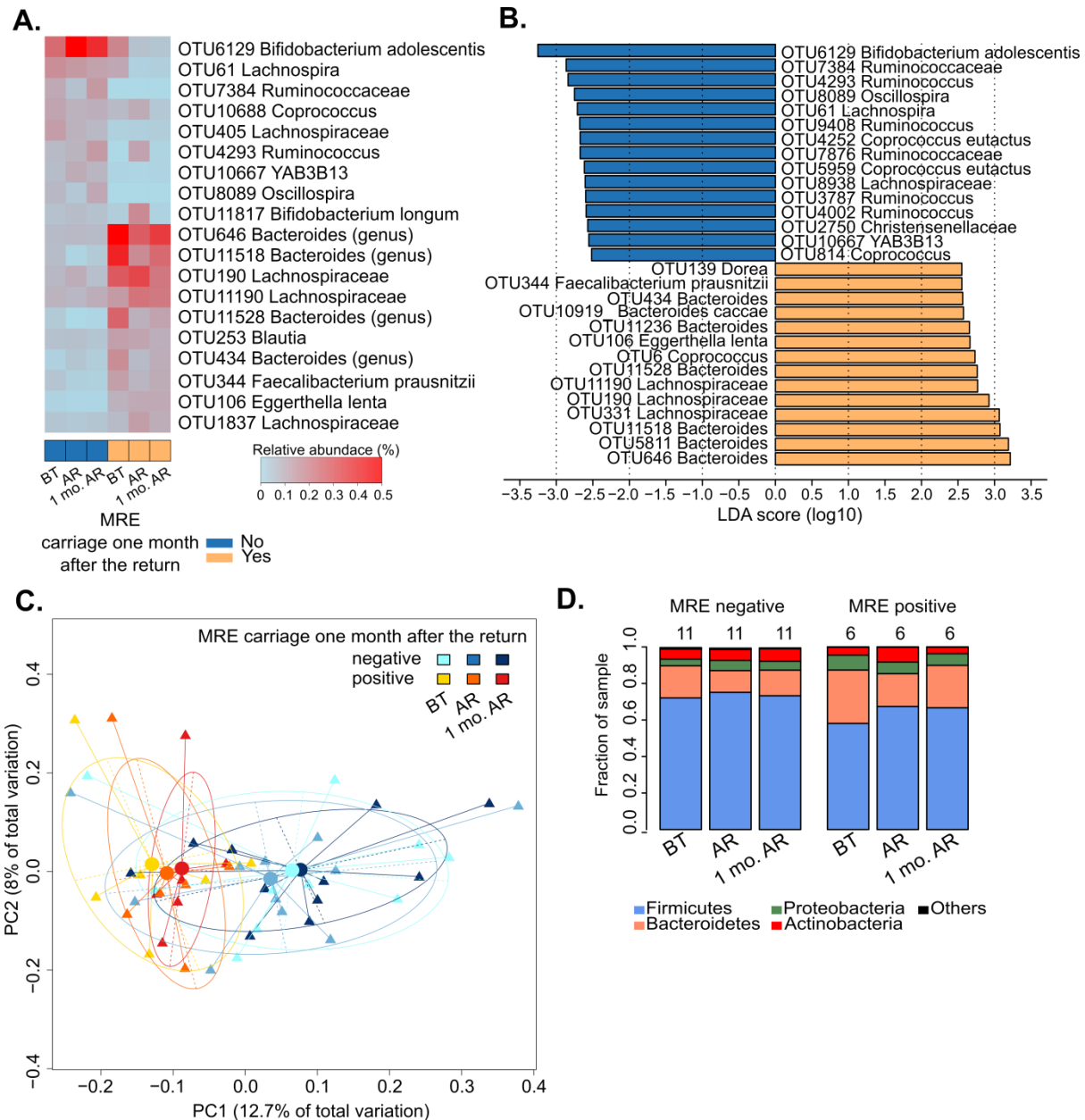
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457 **Figure 1. Microbiota changes with respect to the occurrence of diarrhoea during the travel.** (A)
 458 PCoA plot showing the distribution of samples taken at the return, from travellers who had (red
 459 triangles) or not (blue triangles) diarrhoea during the travel. Percentage of variation explained by the
 460 first two axes is indicated. (B) Boxplots and dot plots depicting the values and their corresponding
 461 distributions of ecological indexes, computed at the 16S OTU level, before travel (BT) and at return
 462 (AR), between travellers experiencing or not diarrhoea. Stars correspond to the following p-values: * =
 463 <math><0.05</math>; ** = <math><0.01</math>; *** = <math><0.001</math>. (C) Bar plots reporting the averaged relative abundance (/100) of
 464 phyla detected by mapping to 16S Greengenes database in travellers without diarrhoea (n=33 of
 465 which 11 were still MRE positive one month after the return) and those with (n=6 of which 3 were still
 466 MRE positive one month after the return). Numbers at the top of the bars indicate the amount of

467 samples for each travel time point/condition. (D) Bar plot reporting log₁₀-transformed LDA scores of
 468 OTUs selected by LEfSe analyses (p-value <0.05). Cohorts of samples and colour label are the same
 469 as explained in (A). Only OTUs with a log₁₀ LDA score of at least 3 are represented. Greengenes
 470 taxonomy identifiers for all OTUs are reported in Supplementary Table 3.

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475 **Figure 2. Comparison of travellers who acquired MRE and remained positive (n=11) or became**
 476 **negative (n=6) one month after return.** (A) Heat map illustrating the mean relative abundance
 477 (expressed as percentage of total reads) of 19 OTUs in the three sampling points (BT, AR and 1 mo.
 478 AR = before travel, at return and one month after the return, respectively) of travellers MRE positive
 479 (yellow bottom bar) and MRE negative (blue bottom bar) one month after the return. OTUs were
 480 selected if the p-value was < 0.05 (Wilcoxon rank sum test), the fold change was of at least 2, and
 481 when the mean relative abundance was of at least 0.1% in one of the six represented conditions
 482 represented (i.e. BT, AR and 1 mo. AR of MRE-positives and of MRE-negatives one month after the
 483 return). Greengenes taxonomy identifiers for all OTUs are reported in Supplementary Table 3. (B)

484 Barplot reporting log₁₀-transformed LDA scores from each OTUs resulting significant (p-value < 0.05)
485 from LEfSe analyses on samples from travellers MRE positive (yellow) and MRE negative (blue) one
486 month after the return. Only OTUs with a log₁₀ LDA score of at least 2.5 were retained for graphic
487 representation. (C) PCoA plot showing the distribution of samples from time points of travellers MRE
488 negative and MRE positive one month after return. PCO1 (12.7%) and PCO2 (8%) represent the
489 degrees of variance of each axis. Samples are grouped in clusters delimited by ellipses. Represented
490 centroids (spheres) capture the origin of each ellipsis. (D) Bar plots reporting the averaged relative
491 abundance (/100) of phyla detected by mapping to 16S Greengenes database in travellers MRE
492 negative (n=11) and MRE positive travellers (n=6) one month after return.
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Table 1. Summary of global PERMANOVA analyses performed on the cohort of 39 travellers at return.

Variables	Conditions	rRNA subunit	Pseudo-F	p-value
Region	Sub-Saharan Africa / Latin America / Asia	16S	0.89	0.75
		23S	0.81	0.82
Type of travel	Organised tour/ Family / Mix of all-inclusive resorts and organised tours / Backpacking / All-inclusive resort	16S	1.08	0.25
		23S	1.11	0.23
Duration of travel (*)	From 1 to 9 weeks	16S	1.04	0.35
		23S	0.82	0.71
Malaria prophylaxis	No / Yes	16S	0.79	0.86
		23S	0.76	0.80
Type of malaria prophylaxis	Atovaquone-proguanil / Chloroquine / Doxycycline / None	16S	0.95	0.57
		23S	0.92	0.63

(*) The test done was DistML (see Methods).

Table 2. Summary of pairwise PERMANOVA tests performed at each travel time point*.

Travel time point	Number of travellers	Variable	rRNA	t	p-value
Before travel	43	Diarrhoea during travel	16S	1.05	0.25
			23S	1.1	0.18
		MRE acquisition	16S	1.09	0.15
			23S	1.05	0.29
At return	39	Diarrhoea during travel	16S	1.44	0.003
			23S	1.8	0.0007
		MRE acquisition	16S	1.12	0.12
			23S	1.13	0.15
One month after the return	17	Diarrhoea during travel	16S	1.04	0.3
			23S	0.97	0.6
		MRE carriage at return	16S	1.21	0.01
			23S	1.16	0.07

The number of samples analysed varied among travel time points according to the use of antibiotics and MRE acquisition. Before travel we have considered all the 43 travellers. At return we analysed only subjects who did not take antibiotics during the travel. One month after the return, we considered travellers who acquired MRE during travel and were not treated with antibiotics after the trip.