Research paper title:

Persistence of wastewater antibiotic resistant bacteria and their genes in human fecal material

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Abstract

Domestic wastewater is a recognized source of antibiotic resistant bacteria and antibiotic resistance genes (ARB&ARGs), whose risk of transmission to humans cannot be ignored. The fitness of wastewater ARB in the complex fecal microbiota of a healthy human was investigated in feces-based microcosm assays (FMAs). FMAs were inoculated with two wastewater isolates, *Escherichia coli* strain A2FCC14 (MLST ST131) and *Enterococcus faecium* strain H1EV10 (MLST ST78), harboring the ARGs bla_{TEM}, bla_{CTX}, bla_{OXA-A} and *van*A, respectively. The FMAs, incubated in the presence or absence of oxygen or in the presence or absence of the antibiotics cefotaxime or vancomycin, were monitored based on cultivation, ARGs quantification and bacterial community analysis.

The fecal bacterial community was dominated by members of the phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia*. The ARGs harbored by the wastewater isolates could be quantified after one week, in FMAs incubated under both aerobic and anaerobic conditions. These observations were not significantly different in FMAs incubated anaerobically, supplemented with sub-inhibitory concentrations of cefotaxime or vancomycin. The observation that ARGs of wastewater ARB persisted in presence of the human fecal microbiota for at least one week supports the hypothesis of a potential transmission to humans, a topic that deserves further investigation.

Keywords:

human fecal microbiota, microcosm assays, microcosm effect, antibiotic resistance transmission, antibiotic resistant bacteria, antibiotic resistance genes.

One sentence summary:

Antibiotic resistance genes harbored by wastewater bacterial isolates persisted in healthy infant´s stool-based microcosms under different conditions, including the presence of sub-inhibitory concentrations of antibiotics.

1. Introduction

Antibiotic resistance, defined as the capability of bacteria to survive and proliferate in the presence of antibiotics, is a natural bacterial property (Davies and Davies 2010). Bacteria resistant to antibiotics, against which were once susceptible, owe that capability to the acquisition of antibiotic resistance genes (ARGs), most of the times through horizontal gene transfer (Bengtsson-Palme *et al.,* 2018; Summers 2006). Acquired antibiotic resistance emergence and proliferation have been attributed to factors such as the presence of antibiotics and other antimicrobials, metals or conditions still unknown, which through their stressor effects exert what has been designated as selective pressures (Martinez 2008; Rosenblatt-Farrell 2009). Ubiquitous bacteria, harboring acquired antibiotic resistance genes, can thrive in the environment, in particular in wastewater, water, soil and wildlife (Berendonk *et al.,* 2015; Huddleston 2014). The paths of transmission of these bacteria back to humans are not fully understood and the probability of such occurrence hardly can be estimated based on the current knowledge (Manaia 2017). A still unanswered question refers to the capability of antibiotic resistant bacteria (ARB) from environmental origin, as well as their genes, to is survive or persist in the human body (Bengtsson-Palme *et al.,* 2018; Larsson *et al.,* 2018; Manaia 2017). Specifically, if it is assumed that the digestive tract is the entry portal, one of the questions would be if these bacteria would be able to survive the competition of complex intestinal microbiome (Manaia 2017; Vaz-Moreira *et al.,* 2014). The role of the digestive tract as a relevant entry portal assumes a particular likeliness in situations of ingestion of raw vegetable-based food products, therefore acting as potential sources of ARB to humans

(Holzel *et al.*, 2018; Valerio *et al.*, 2006; Zhang *et al.*, 2019). The risks of ARB occurrence in vegetables can be enhanced in scenarios of manure soil amendment or water reuse for irrigation (Becerra-Castro *et al.*, 2015; Heuer *et al.*, 2011; Marti *et al.*, 2013). Indeed, several recent studies have shown the presence of ARB and ARGs in raw-eaten products (e.g. lettuce), raising concerns for consumers, particularly for immunocompromised people (Araujo *et al.*, 2017; Blau *et al.*, 2018; O'Flaherty *et al.*, 2019; Zhang *et al.*, 2017; Zhu *et al.*, 2017). However, even after ingestion, the success of allochthonous bacteria in the intestinal tract is supposedly antagonized by the gut microbiota, a complex and dynamic community of microorganisms colonizing the gastrointestinal tract of humans since birth (Donaldson *et al.*, 2016; Gibson *et al.*, 2014). While indigenous (or autochthonous) microorganisms may have the intestine as a long term or almost permanent niche, allochthonous microorganisms may colonize transiently the human gut, although some, the most fitted, will be able to persist for long time periods (Milani *et al.*, 2017; Ventura *et al.*, 2009). Among the most fitted bacterial groups, it can be hypothesized that bacteria of enteric origin, like *Escherichia coli* or *Enterococcus* spp., are good candidates to survive the competition of the fecal microbiome of a healthy individual. These can certainly be part of the 10^6 to 10^9 bacterial cells that, depending on the different dietary intake, can be ingested daily (Derrien and van Hylckama Vlieg 2015; Lang *et al.*, 2014). If part of these bacteria carry acquired ARGs, such fact can hypothetically increase their fitness in the presence of antibiotics and/or facilitate the interchange of those genes with the native community (Salyers *et al.*, 2004). Therefore, the contamination of the human food chain with environmental ARB might represent a risk for the subsequent transmission to humans. Among the multiple bottlenecks that may hamper the successful human-gut colonization by allochthonous bacteria, are the capacity to survive the complex native microbiome and/or the stability of the respective ARGs.

These questions boosted this study that used the fecal material of a healthy infant as a model of human gut microbiota to assess the persistence of enteric ARB isolated from wastewater, *Escherichia coli* strain A2FCC14 and *Enterococcus faecium* strain H1EV10, harboring the ARGs bla_{TEM}, $bla_{CTX\text{-}M}$, $bla_{OXA\text{-}A}$ and *van*A, respectively. Specifically, the objectives of this work were to assess: 1) if those wastewater ARB and the respective ARGs were outcompeted in the presence of the human fecal microbiota, 2) if the cell-free ARGs could persist in that environment, and 3) the influence of the presence of oxygen or of antibiotics on the survival and persistence of the ARB and ARGs measured in 1) and 2). Assuming successful colonization, for which it is necessary that the exogenous enteric bacteria can thrive in the presence of fecal material, an additional question is if the acquired ARGs will be lost because represent a fitness cost.

2. Material and methods

The survival of wastewater antibiotic resistant isolates and persistence of the respective ARGs was assessed in the presence of the complex human fecal microbial community. The experiments were conducted in FMAs composed of human stool specimens spiked with two wastewater isolates: *E. coli* strain A2FCC14 (harboring the ARGs bla_{TEM}, bla_{CTX} and $bla_{OXA\text{-}A}$) and *Ent. faecium* strain H1EV10 (harboring the ARG *van*A), or with the respective DNA extracts. The environmental variables tested in the FMAs were aerobic *vs*. anaerobic conditions, and the effect of single or multiple doses of sub-inhibitory concentrations of cefotaxime or vancomycin, under anaerobic conditions. The FMAs were incubated at 37 ºC, and samples were collected at 0, 1, 3 and 7 days. Monitoring was based on the enumeration of culturable bacteria, quantitative PCR (qPCR) analysis of antibiotic resistance and 16S rRNA genes and bacterial community analyses based on 16S rRNA gene amplicon sequencing.

135

136 *2.1. Bacterial strains*

137 The strains *E. coli* A2FCC14 (isolated from raw municipal wastewater) and *Ent. faecium*
138 H1EV10 (isolated from untreated hospital effluent) (Varela *et al.*, 2013) were used to
139 inoculate the fecal microcosm assays (FMAs). The Whole Genome Shotgun projects of
140 strains A2FCC14 and H1EV10 have been deposited at DDBJ/ENA/GenBank under the
141 accession numbers WSZB00000000 and WSZC00000000, respectively. These strains
142 have an Average Nucleotide Identity (ANI) of orthologous gene pairs shared between two
143 microbial genomes with the type strains of the species of 98.5% and 99.5%, respectively.
144 ARGs conferring resistance to beta-lactam (bla_{TEM}, $bla_{\text{CTX-M}}$, and $bla_{\text{OXA-A}}$), harbored by
145 the *E. coli* strain and to vancomycin (*van*A) harbored by the *Ent. faecium* strain were
146 monitored in FMAs. For inoculum preparation, both strains were handled as follows: *E.*
147 *coli* A2FCC14 was cultivated on m-FC agar medium (fecal coliform agar, Difco BD)
148 supplemented with cefotaxime 4 mg L^{-1} and incubated overnight at 37 °C and *Ent.*
149 *faecium* H1EV10 was cultivated on m-Enterococcus agar medium (Difco BD)
150 supplemented with vancomycin 16 mg L^{-1} and incubated for 48 hours at 37 °C. The
151 biomass collected from each of those bacterial cultures was used to prepare a bacterial
152 suspension in saline solution (0.85% (w/v) NaCl) with a cell density of approximately
153 10^8 and 10^7 Colony Forming Units (CFU) mL^{-1} for *E. coli* A2FCC14 and *Ent. faecium*
154 H1EV10, respectively. This cell density was in accordance with the average density of
155 bacteria of these groups in the human microbiome and although higher than can be
156 expected from a possible ingestion and digestion, it overcomes the experimental risk of
157 reaching the values below the limits of quantification. After an initial calibration of CFU
158 versus turbidity at 610 nm, the cell suspensions used to inoculate each FMA were
159 measured by spectrophotometry.

160
161 *2.2. Feces-based microcosm assays (FMAs)*

162 The FMAs prepared with fecal material of a healthy child were used as a model to assess
163 the fate of ARB and ARGs. All assays used fecal material supplied by a single healthy
164 donor, aged 40 to 58 months during this study, and who was never submitted to
165 antibiotherapy. The option for a donor with these characteristics was because although
166 the gut microbiota was certainly affected by diet and lifestyle, it was not rearranged due
167 to previous antibiotic exposure, a control considered relevant for the present experimental
168 design. In total, were collected twelve stool samples, each used to run an independent
169 FMA (Table 1). For each FMA experiment, were collected ≥ 80 g of fecal material that
170 was stored at 4 °C for no more than 3 days. Each FMA comprised inoculated assays (M-
171 assays) and the respective non-inoculated controls (C-assays). FMAs were tested under
172 aerobic and anaerobic conditions or under anaerobic conditions spiked with antibiotics.
173 The evaluation of the effect of oxygen was considered of interest given the fact that *E.*
174 *coli* are facultative anaerobes and enterococci are aerotolerant, supporting some inference
175 about the influence of the fitness of these exogenous bacteria versus the effect of
176 competition by the fecal microbiota. In total, four FMAs were incubated under aerobic
177 conditions [40 (C and M); 44 (C and M); 48 (C and M); 50 (C and M)], three under
178 anaerobic conditions [50 (C and M); 54 (C and M); 58 (C and M)] and four under
179 anaerobic conditions, spiked with subinhibitory concentrations of antibiotics. Antibiotic
180 spiking was done in a single-dose [54-C+cefotaxime (54-C.C), 54-C+vancomycin (54-
181 C.V), 54-M+cefotaxime (54-M.C) and 54-M+vancomycin (54-M.V)] or in multiple-
182 doses [58-C+cefotaxime (58-C.C), 58-C+vancomycin (58-C.V), 58-M+cefotaxime (58-
183 M.C) and 58-M+vancomycin (58-M.V)] (Table 1). Anaerobic FMAs were handled and
184 incubated in an anaerobic chamber (Whitley Workstation A35, containing a gas mixture
185 of 85% carbon dioxide, 10% nitrogen, 5% hydrogen). Each experimental set (FMA)

comprised 24 vial assays, corresponding to triplicates of spiked and non-spiked assays to be sacrificed for analyses after 0, 1, 3 and 7 days of incubation (3 replicates x spiked/non-spiked x 4 incubation periods). Although the transit time of bacteria in the large intestine is estimated to be ~2.5 days, ingested bacteria can be detected in the intestine for one week (Berg 1996; Derrien and van Hylckama Vlieg 2015). Based on this note and preliminary assays, an incubation period of 7 days (extended to 30 days for occasional analysis) was selected.

To prepare these experimental sets, stool samples were diluted five times with sterile saline solution (0.85% (w/v) NaCl) and divided in 24 aliquots of 15 mL each. Half of these 24 aliquots were inoculated with 2 mL of a bacterial cocktail, described in the previous section, composed by the mixture of both strains, *E. coli* A2FCC14 and *Ent. faecium* H1EV10 (M-assays). The other half of the aliquots, corresponding to non-inoculated controls (C-assays), was spiked with 2 mL of sterile saline solution. FMAs 40, 44 and 48 were incubated aerobically. FMA50 comprised two parallel FMAs (24 vials incubated aerobically and 24 anaerobically). FMA54 and FMA58 were incubated anaerobically and were spiked with antibiotics. Briefly, FMA54 comprised 24 vials without antibiotic, 24 spiked with one dose of cefotaxime (4 mg L^{-1} for each microcosm) and 24 spiked with one dose of vancomycin (16 mg L^{-1} for each microcosm). FMA58 differed from FMA54 on the method of antibiotic spiking which in FMA58 was supplied at three antibiotic moments (3 x 4 mg L^{-1} for cefotaxime or 3 x 16 mg L^{-1} for vancomycin) at time 0, 1 day and 3 days, before each sample collection. Cell-free DNA supplemented FMAs, FMA44 and FMA48, were spiked with DNA extracts from *E. coli* A2FCC14 and *Ent. faecium* H1EV10. The DNA fragment length of the DNA extract of *E. coli* A2FCC14 was assessed in an agarose gel (1 %) electrophoresis, originating a single band with a molecular weight 10-50 000 bp, with no smearing effect, which would suggest that DNA

was degraded. The quantity of DNA used was 2.0 and 1.5 µg of DNA extracted from *E. coli* A2FCC14 and *Ent. faecium* H1EV10, corresponding to the cell density used in the ARB inoculated FMAs (Table 1). FMA44 was designed aiming at assessing the persistence of the spiked free DNA and FMA48 aimed at assessing the potential occurrence of natural transformants, able to uptake the free DNA. Therefore FMA48 was composed of 3 separated assays (6 vial assays corresponding to triplicates of non-spiked assays, sampled at T0 and T7, 6 vial assays corresponding to triplicates of bacteria-spiked assays, sampled at T0 and T7 and, 6 vial assays corresponding to triplicates of assays spiked with cell-free DNA extracted from the test bacteria, sampled at T0 and T7). Natural transformants were tentatively isolated on the m-FC or m-Enterococcus culture media supplemented with antibiotic (cefotaxime or vancomycin, respectively). Cultivable bacteria counts were processed immediately, and aliquots for dry weight determination and total DNA extraction were stored at -20 and -80 ºC, respectively, until used. All determinations were done in triplicate. The stool dry weight was determined in 1 mL fecal slurry samples by incubation at 60 ºC, until a constant weight was reached, which corresponded to approx. 5 days. This study was approved by the Ethics Committee of the Universidade Católica Portuguesa in Porto.

2.3. Enumeration of cultivable bacteria in FMAs

Luria-Bertani Agar (LA) (Invitrogen), m-FC agar (Difco BD) and m-Enterococcus agar (Difco BD) were used for enumeration of total heterotrophic bacteria (HB), enterobacteria and enterococci, respectively. When necessary, these culture media were supplemented with cefotaxime (4 mg L^{-1}; Sigma-Aldrich, St Louis, USA) or vancomycin (16 mg L^{-1}; Sigma-Aldrich, St Louis, USA), at concentrations corresponding to minimum inhibitory concentration (MIC) for *E. coli* or enterococci (CLSI 2014). For bacterial enumeration,

volumes of 1 mL were collected from each FMA, serially diluted in sterile saline solution (0.85% (w/v) NaCl) and plated on the adequate culture medium using the Miles and Misra method (Miles *et al.*, 1938). Cultures were incubated at 37 ºC for 24 h on LA and m-FC, or 48 h on m-Enterococcus agar. All bacterial counts were performed in triplicate.

2.4. DNA extraction

Total DNA was extracted from 1 mL of fecal slurry (corresponding to approximately 240 mg of wet sedimented stool) using the NZY Tissue gDNA Isolation kit (Nzytech, Portugal) according to the manufacturer's instructions. The DNA concentration in the extracts was quantified using Qubit fluorometer (Thermo Fisher Scientific, USA). Extracts were preserved at -20 ºC until qPCR or microbial community analyses were performed.

2.5. Quantitative PCR

The abundance of the genes bla_{TEM}, bla_{CTX}, $bla_{\text{OXA-A}}$ and *van*A, harbored by the inoculated bacteria, was monitored based on real-time quantitative PCR (qPCR). The total bacterial abundance was assessed based on the housekeeping gene 16S rRNA. The qPCR was conducted in a StepOne™ Real-Time PCR System (Life Technologies, Carlsbad, CA) following the conditions previously described (Narciso-da-Rocha *et al.*, 2018) for the ARGs bla_{TEM}, bla_{CTX} and $bla_{\text{OXA-A}}$ and the 16S rRNA gene. The qPCR conditions for the detection of the gene *van*A were set up in 25 µL volume using the Power SYBR Green mastermix (Thermo Fisher Scientific, Austin, USA) containing 10 µM of each primer: VnF 5'-ATCGGCAAGACAATATGACAGC-3' and VnR 5'-AGCCTGATTTGGTCCACCTC-3' (Lata *et al.*, 2009). The PCR program was initiated by a period of 5 min at 95 °C, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 30

sec. The standard curve for *van*A gene was prepared using a clone of the *van*A gene of *Ent*. *faecium* H1EV10 with an efficiency between 96 and 105%.

2.6. Bacterial community analysis

The bacterial community composition of FMA40, 44, 50, 54 and 58 was analyzed at time zero (T0) and after 7 days of incubation (T7), based on the hypervariable region V3/V4 of the 16S rRNA gene, using paired-end Illumina MiSeq® Sequencing (Genoinseq, Portugal) as previously described by Narciso-da-Rocha *et al.* (2018). The primers used were forward primer Bakt_341F 5'- CCTACGGGNGGCWGCAG -3' and reverse primer Bakt_805R 5'- GACTACHVGGGTATCTAATCC -3' according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Demultiplexed raw reads were extracted from Illumina MiSeq® System in fastq format and the reads were processed and analyzed using Quantitative Insights Into Microbial Ecology (QIIME2) pipeline (version 2017.10; http://qiime2.org/) (Bolyen *et al.,* 2018). Sequences shorter than 200 bp and with average quality scores lower than 25 were eliminated. Sequences with average quality lower than 25 in a window of 5 bases were trimmed using the software PRINSEQ (Schmieder and Edwards 2011). Sequences were filtered, merged and, chimeric reads were removed by the DADA2 software package enclosed in QIIME2 (Callahan *et al.,* 2016). Taxonomy was assigned to the amplicon sequence variants (ASVs), sequences with 100% identity, using the ARB SILVA database release 132 (Yilmaz *et al.,* 2014). A total of 4 750 244 reads (ranging from 22 176 to 112 134 reads per sample) and 1714 ASVs (ranging from 171 to 263 per sample) were obtained from the 105 datasets, corresponding to triplicates of 35 samples of C and M FMAs.

2.7. Statistical analyses

Cultivable bacteria counts were expressed as log values of colony-forming units (CFUs) per g of stool dry weight . Gene abundance was expressed as gene copy number per g of dry weight (abundance) or per 16S rRNA gene copy number (relative abundance or prevalence). One-way analysis of variance (ANOVA), Tukey's and Bonferroni post-hoc tests and *t*-test (SPSS Statistics for Windows v.24.0; IBM Corp., Armonk, NY, USA) were used for determination of statistically significant differences ($p < 0.01$) of cultivable bacteria counts, abundance and prevalence of measured genes when comparing different incubation times or conditions. The bacterial community composition was expressed as the relative abundance of reads number of a specific bacterial group per total reads number. Correlations between the relative abundance of bacterial groups at phylum and family level were analyzed using the statistical analysis of taxonomic and functional profiles using the software STAMP v2.1.3 and Canoco 5.01 (Parks *et al.,* 2014; Šmilauer and Lepš 2014). Statistically significant differences between bacterial phyla or family relative abundances were determined using a two-tailed *t*-test ($p <0.01$) and the p values were corrected for multiple testing using the Benjamini-Hochberg FDR (Benjamini and Hochberg 1995).

3. Results and discussion

3.1. Microbial community composition of the fecal material

Since the fecal microbiota composition and the respective temporal variations could somehow influence the fate of exogenous ARB and ARGs, the analysis of the fecal bacterial community was necessary in this study. This part of the work had two aims, assess the phylogenetic diversity in the fecal material and assess the variations that were due to the microcosm effect. Over the study period, the infant (40-58 months) fecal microbiota was dominated by members of the bacterial phyla *Firmicutes* (41.6-47.1%)

311 and *Bacteroidetes* (23.5-36.2%), followed by *Actinobacteria* (7.2-22.5%),
312 *Proteobacteria* (1.5-7.4%) and *Verrucomicrobia* (1.2-7.4%) (Table S1). These results are
313 in line with similar studies involving healthy individuals in the same age range (Monira
314 *et al.*, 2011). Statistically significant variations were observed over the study period for
315 all the above mentioned phyla, although none with a clear trend of increase or decrease.
316 These variations might be due to diet and/or the natural dynamic processes observed in
317 infants gut microbiota (Milani *et al.*, 2017; Zmora *et al.*, 2019). Although these variations
318 were smooth and with little expected impacts on the survival of the exogenous bacteria,
319 whenever adequate they will be used to discuss the results.
320
321 *3.2 Effect of incubation condition on the microbial community composition*
322 The microcosm effect, meaning the bacterial community variations that occurred during
323 the incubation period (7 days), which could allegedly influence the survival or persistence
324 of exogenous bacteria or their ARGs, was assessed in non-inoculated FMAs, under both
325 aerobic and anaerobic conditions. Besides the variation of phyla composition, it was also
326 assessed the variation in the relative abundance of members of the families that include
327 the wastewater ARB surrogates used in the inoculated FMAs, *Enterobacteriaceae* and
328 *Enterococcaceae* (Table 2). The relative abundance of *Proteobacteria* significantly
329 ($p<0.01$) increased (ratio T7/T0 > 1) during the incubation under aerobic, but not under
330 anaerobic conditions (Table 2). It was also observed that the relative abundance of
331 *Enterobacteriaceae* in the non-inoculated assays was always higher than in inoculated
332 microcosms (Table 2), which might be due to a steady-state-like for *Enterobacteriaceae*
333 in the fecal microbial community, in which these bacteria, in equilibrium with the
334 remaining community, are kept at a certain level and eventually exogenous bacteria, as
335 were the wastewater isolates in this case, may have a limited proliferation capacity.

The relative abundance of the bacterial phylum *Firmicutes* was fairly stable over the 7 days period, under both aerobic and anaerobic conditions, with the ratio T7/T0 varying between 0.65 and 0.99 in non-inoculated FMAs (Table 2). Curiously, members of family *Enterococcaceae*, of the phylum *Firmicutes*, were not detected in non-inoculated FMAs. The observation that anaerobic conditions had a lower impact on the microcosm effect than aerobic conditions, mainly in the groups that include the surrogates used, recommended that the effect of antibiotics would be better examined under anaerobiosis. In the FMA54 and FMA58, the addition of cefotaxime or vancomycin only affected significantly ($p<0.01$) the relative abundance of *Proteobacteria* (Table S3). Curiously, this effect was different when a single- (FMA54) or multiple-antibiotic-dose (FMA58) was used (Table S3). A single cefotaxime dose (54-C.C and 54-M.C) significantly ($p<0.01$) reduced the relative abundance of *Proteobacteria* in both inoculated and non-inoculated FMAs (ratio antibiotic/no antibiotic < 1). In contrast, multiple cefotaxime doses led to a significant ($p<0.01$) reduction on the relative abundance of *Proteobacteria* only in inoculated FMAs (58-M.C). The use of a single vancomycin dose significantly ($p<0.01$) reduced the relative abundance of *Proteobacteria* in the non-inoculated samples (54-C.V; Table S3). In contrast, three doses of vancomycin led to a significant ($p<0.01$) increase of the *Proteobacteria* relative abundance, only in non-inoculated FMA (58-C.V). As noted above for oxygen, also the antibiotic effect on members of the *Enterobacteriaceae* followed the same pattern as for the *Proteobacteria* phylum (Table S3). In contrast, no noticeable effects of antibiotics were observed on *Enterococcaceae* in inoculated FMAs.

These different behaviors of the bacterial community in presence of a single or multiple doses of antibiotics may suggest the rearrangement of the community or the charity among community members that able to degrade the antibiotics. The Principal

Component Analysis (PCA) of the non-inoculated assays suggest that irrespective of the antibiotic and spiking method, members of the families *Burkholderiaceae* (phylum *Proteobacteria*) and *Lachnospiraceae* (phylum *Firmicutes*) were those whose relative abundance decreased during the incubation period. The contribution of these groups to the community rearrangement may have been exacerbated in the presence of antibiotics (Figure S1 A, B, C, and D). In general, the results suggest that antibiotics might have important effects on the fecal bacterial community composition and structure, with effects being noticed in the relative abundance of members of the phyla *Firmicutes*, *Actinobacteria* and *Bacteroidetes* (Table S3). The relative abundance of *Actinobacteria* was significantly lower in the fecal material used in the FMA where a single antibiotic dose was tested (FMA 54) than in that where three successive doses were assayed (FMA 58). The opposite was observed for the relative abundance of *Bacteroidetes* (Table S1). Since both phyla members were observed to have strong correlation with antibiotic spiking, the distinct behavior observed in response to one or multiple antibiotic doses may be also a result of distinct microbiota composition. The importance of these bacterial groups as potential barriers or facilitators for antibiotic-induced bacterial community rearrangements may deserve further investigation. In general, the PCA results (Figure S1) are in agreement with the literature that suggests that the oral or intravenous antibiotic administration can promote the disturbance of the human gut microbiome (Bhalodi *et al.*, 2019; Francino 2016). The PCA analysis (Figure S1) suggests that the significant variations observed in *Proteobacteria* relative abundance (Table S3), might be due to complex fecal bacterial community rearrangements rather than to the increase or decrease of a specific bacterial lineage. Indeed, it is this type of indirect effect that was demonstrated for vancomycin, a glycopeptide antibiotic used against Gram-positive bacterial infection. In that case, it was associated with the decrease of *Firmicutes* that led

386 to the increase of *Proteobacteria* in the fecal microbial community (Isaac *et al.*, 2017;
387 Vrieze *et al.*, 2014). Indeed, although there is no evidence specifically for vancomycin
388 and cefotaxime, the ability of sub-inhibitory concentrations of antibiotics to modify the
389 competition between bacterial species within a microbial community has been discussed
390 (Hall and Corno 2014; Martinez 2009).
391
392 *3.3. Monitoring of culturable bacteria and antibiotic resistance genes*
393 The native and exogenous culturable populations of enterobacteria, enterococci and/or
394 total heterotrophic bacteria were monitored over time and under distinct conditions.
395 Heterotrophic and enterobacteria counts presented similar variation patterns in inoculated
396 and non-inoculated FMAs and, in both cases, it was possible to infer about the beneficial
397 oxygen effect on the first day of incubation, characterized by a slight CFU increase.
398 Therefore, it is suggested that the fitness of enteric bacteria cannot be explained based
399 only on the fecal microbiota competition, it is also explained by the survival capacity, in
400 this case, higher in the presence of oxygen (Figure 1). In contrast, the behavior of the
401 aerotolerant enterococci was identical in the presence or absence of oxygen. Also,
402 enterococci counts presented a similar variation pattern in inoculated and non-inoculated
403 assays (Figure 1). In general, under anaerobic conditions, enterococci, enterobacteria and
404 heterotrophic bacteria counts presented in average reductions of 1.1, 1.0 and 0.1 log units
405 over the 7 incubation days, respectively (Figure 1). However, the stochastic nature of
406 these variations is suggested, when different FMAs are compared (Figure 1).
407 The exogenous bacteria were wastewater isolates, *E. coli* A2FCC14 and *Ent. faecium*
408 H1EV10, belonging to the multilocus sequence types ST131 and ST78, respectively,
409 therefore genetically related with widespread pathogens (Khan *et al.*, 2010; Nicolas-
410 Chanoine *et al.*, 2014). These exogenous bacteria were traced based on their ARGs

411 *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{OXA-A}$ and *van*A (Figure 2). Under aerobic conditions, the 16S rRNA
412 gene abundance per gram of feces dry weight, decreased during the incubation period on
413 average 0.26 log-units in inoculated FMAs (40-M and 50-M) and 0.32 log-units for non-
414 inoculated FMAs (40-C and 50-C). These values were comparatively higher under
415 anaerobic conditions, of 0.48 log-units in inoculated FMAs (50-M, 54-M, and 58-M) and
416 of 0.42 log-units in non-inoculated FMAs (50-C, 54-C and 58-C).
417 Among the analyzed ARGs, only *bla*$_{TEM}$ was detected in non-inoculated microcosms
418 (Figure 2). The *bla*$_{TEM}$ gene may have been ingested by the donor (e.g. fresh produce)
419 (Blau *et al.*, 2018), and its occurrence in the gut microbiota of healthy individuals, even
420 if never exposed to antibiotics, has been reported (Fouhy *et al.*, 2014; Sommer *et al.*,
421 2009). In general, the *van*A gene abundance (per gram of dry weight) did not vary
422 significantly from T0 to T7, with average measurements at T0 and T7 of 5.87 ± 0.11 and
423 5.79 ± 0.15 log-units under aerobic conditions, and 7.34 ± 1.17 and 7.17 ± 1.12 log-units
424 under anaerobic conditions (Figure 2). In contrast, occasionally, although under both
425 aerobic and anaerobic conditions (40-M and 58-M, Figure S2) the prevalence of *van*A
426 (per 16S rRNA gene) increased significantly ($p<0.01$), which may be due to the decrease
427 of the overall bacterial population, herein measured in 16S rRNA gene abundance.
428 Aerobically, on average (FMAs 40-C, 50-C, 40-M and 50-M), the abundance of *bla*$_{TEM}$
429 gene had a significant ($p<0.01$) increase from 7.13 ± 0.55 log-units at T0 to 8.23 ± 0.44
430 log-units at T7 (Figure 2). Anaerobically, *bla*$_{TEM}$ was not detected in one of the non-
431 inoculated assays (58-C) and it decreased significantly ($p<0.01$) in the FMA50 (50-C, 50-
432 M; Figure 2). In the FMA54, *bla*$_{TEM}$ gene increased and decreased significantly ($p<0.01$)
433 in the non-inoculated and inoculated assays, respectively (54-C, 54-M; Figure 2). On the
434 average of the FMAs (50-C, 54-C, 50-M, 54-M, and 58-M), its abundance varied from
435 7.61 ± 1.12 log-units at T0 to 8.18 ± 0.74 log-units at T7 (Figure 2). The prevalence of

436 the *bla*$_{TEM}$ gene (per 16S rRNA gene) followed the same pattern of variation of the *bla*$_{TEM}$
437 gene abundance along time, which suggests that the variations of *bla*$_{TEM}$ gene are mainly
438 due to total bacteria variations (Figure 2, Figure S2).
439 The abundance (per gram of dry weight) of the *bla*$_{CTX-M}$ gene increased significantly
440 ($p<0.01$) under aerobic incubation (40-M; Figure 2) ranging from 6.83 ± 0.15 to 7.80 ±
441 0.05 log-units and, the same gene decreased significantly ($p<0.01$) under anaerobic
442 incubation (50-M; Figure 2) varying from 7.04 ± 0.21 to 5.63 ± 0.46 log-units. Similar
443 results were observed for *bla*$_{OXA-A}$ gene, which abundance (per gram of dry weight) varied
444 significantly ($p<0.01$) from 6.37 ± 0.22 to 7.49 ± 0.06 log-units under aerobic conditions
445 (40-M; Figure 2) and from 6.68 ± 0.14 to 5.75 ± 0.42 log-units under anaerobic conditions
446 (50-M; Figure 2).
447 In general, it was observed that the abundance of the beta-lactamase genes increased
448 under aerobic conditions and decrease under anaerobic conditions (Figure 2). This result
449 that is aligned with the enumeration of culturable bacteria, suggests that the fitness of the
450 exogenous bacteria, and not only the competition by the native microbiota, may dictate
451 the fate of enterobacteria in the FMAs. The prevalence (expressed per 16S rRNA gene
452 copy number) of both *bla*$_{CTX-M}$ and *bla*$_{OXA-A}$ increased under aerobic (40-M, 50-M) and
453 anaerobic conditions (54-M, 58-M, except 50-M) (Figure S2). Comparing the FMA-40
454 and FMA-50 the abundance and prevalence of the *bla*$_{CTX-M}$ and *bla*$_{OXA-A}$ follows the same
455 pattern suggesting that the variations of the beta-lactamase genes are mainly due to
456 bacterial host variations (Figure 2, Figure S2).
457 To test the hypothesis that the fate of the exogenous ARGs is mainly dictated by the
458 survival and integrity of the host cell, cell-free DNA extracts were used for spiking the
459 FMAs (FMA44). The ARGs *bla*$_{CTX-M}$, *bla*$_{OXA-A}$, and *van*A, detected after inoculation at
460 doses of 4-5 log units were below the detection limit after 1 day of incubation (Figure 2),

461 suggesting the rapid DNA degradation by the native fecal microbiota (e.g. extracellular
462 enzymes or bacteria feeding in naked DNA). This result supported the hypothesis that
463 viable or at least integer bacterial hosts are necessary to ensure the ARGs persistence.
464 Moreover, any attempts to isolate transformants from free-DNA spiked FMA48 were
465 unfruitful, therefore failing the evidence of antibiotic resistance acquisition by
466 transformation.
467
468 *3.3.1 Antibiotics effect on exogenous antibiotic resistant bacteria*
469 Culturable enterococci showed a different pattern of variation in inoculated and non-
470 inoculated assays spiked with vancomycin, although in both was observed a significant
471 ($p<0.01$) decrease along time (Figure 1). In the presence of a single-dose of cefotaxime
472 (FMA54; Figure 1), culturable enterobacteria in inoculated and non-inoculated FMAs
473 undergone a significant ($p<0.01$) decrease of approximately 2 log-units after 7 days. A
474 similar, although less intense effect, was observed after administration of a multiple-dose
475 of cefotaxime, with enterobacteria reductions of approximately 1 log-unit in inoculated
476 and non-inoculated microcosms after a week (Figure 1). Generally, in the presence of low
477 concentrations of cefotaxime or vancomycin, heterotrophic bacteria counts presented a
478 similar trend along the time in both inoculated and non-inoculated microcosms (Figure
479 1). Using a single-dose of cefotaxime (FMA 54-M.C, 54-C.C; Figure 1) or vancomycin
480 (FMA 54-M.V, 54-C.V; Figure 1), heterotrophic bacteria counts of inoculated and non-
481 inoculated samples decreased significantly ($p<0.01$) of approximately 3 or 1 log-units,
482 respectively, after 7 days. A similar, albeit less intense effect, was observed after
483 administration of a multiple-dose of cefotaxime (FMA 58-M.C, 58-C.C; Figure 1) where
484 heterotrophic bacteria counts of inoculated and non-inoculated assays decreased
485 significantly ($p<0.01$) of approximately 2 log-units after 7 days.

The abundance of the 16S rRNA gene per gram of feces dry weight, a measure of bacterial abundance, was not significantly different in inoculated and non-inoculated assays, and decreased significantly ($p<0.01$) over the incubation period, in inoculated (from 11.45 ± 0.19 to 10.88 ± 0.28 log-units; FMA 54-M.C, 54-M.V; FMA 58-M.C and 58-M.V) and in non-inoculated microcosms (from 11.38 ± 0.11 to 10.89 ± 0.11 log-units; FMA 54-C.C, 54-C.V; FMA 58-C.C and 58-C.V), irrespective of the use of one or three doses of antibiotic (Figure 3). In the presence of one or three doses of cefotaxime or vancomycin, the abundance of *vanA* gene did not change significantly (FMA 54-M.C, 54-M.V; FMA 58-M.C and 58-M.V; Figure 3). However, in the presence of one or three doses of cefotaxime the *van*A per 16S rRNA gene copy number (prevalence) increased significantly ($p<0.01$) on average from -3.66 ± 0.04 to -3.27 ± 0.08 log-units in inoculated microcosms (FMA 54-M.C and FMA 58-M.C; Figure S3). Similarly, in the presence of three doses of vancomycin, the prevalence (per 16S rRNA gene) of *van*A increased significantly ($p<0.01$) from -3.51 ± 0.06 to -2.95 ± 0.01. Since culturable enterococci decreased over this period, the observed increase is probably due to the sharp decrease of bacteria (16S rRNA gene abundance) observed during incubation. In the presence of one-dose of cefotaxime, the abundance (per dry weight) of the ARGs bla_{TEM}, $bla_{\text{CTX-M}}$ and $bla_{\text{OXA-A}}$ decreased significantly ($p<0.01$) (FMA 54-M.C; Figure 3) while in the presence of three-doses of cefotaxime, the abundance of these genes did not vary significantly (FMA 58-M.C; Figure 3). The prevalence (per 16S rRNA gene) of the ARGs bla_{TEM}, $bla_{\text{CTX-M}}$ and $bla_{\text{OXA-A}}$ increased significantly ($p<0.01$), but only when three doses of cefotaxime were supplied (FMA 58-M.C; Figure S3).

In the presence of one or three doses of vancomycin, the abundance (per dry weight) of the ARGs bla_{TEM} significantly ($p<0.01$) decreased and of $bla_{\text{CTX-M}}$ significantly ($p<0.01$) increased, while the gene $bla_{\text{OXA-A}}$ no significant variations were observed (FMA 54-M.V

511 and FMA 58-M.V; Figure 3). The prevalence of *bla*$_{TEM,}$ *bla*$_{CTX-M}$ and *bla*$_{OXA-A}$ increased
512 significantly ($p<0.01$) only in the presence of three doses of vancomycin (FMA 58-M.V;
513 Figure S3). The abundance and prevalence of the genes *bla*$_{TEM}$ and *bla*$_{CTX-M}$ followed the
514 same pattern of variation when vancomycin was supplemented at three doses suggesting
515 that the variations of these two genes are mainly due to bacterial host variations.
516 Nevertheless, the prevalence increment of the gene *bla*$_{OXA-A}$ in the presence of three-
517 doses of vancomycin suggests that this antibiotic might favor the survival or the
518 persistence of bacteria harboring the *bla*$_{OXA-A}$ gene along the period of incubation.
519
520 *3.4 Relationship between bacterial community and antibiotic resistance genes*
521 To unravel the possible relationship between ARGs persistence and the variation of the
522 fecal bacterial community in the presence of a single- or multiple-dose of cefotaxime or
523 vancomycin, a Canonical Correspondence Analysis (CCA) was performed. This analysis
524 confirmed the microbiota rearrangements due to the microcosm effect (Table 2) by the
525 separation between T0 and T7 samples and highlighted the effects of antibiotics (Table
526 S3, Figure S1) (T7 vs T7.C or T7.V, Figure 4). The use of a single- or multiple- antibiotic
527 doses highlighted distinct patterns of correlation between the fecal bacterial community
528 composition and the quantified genes. While with a single-dose, the ARGs variation was
529 co-linear, with all genes showing the same pattern of variation (Figure 4 A and B), for
530 multiple-doses were observed distinct patterns of variation (Figure 4 C and D). With a
531 single cefotaxime or vancomycin dose, the quantified genes were correlated with groups
532 observed to decrease over the incubation period such as *Ruminococcaceae* and
533 *Burkholderiaceae* (for cefotaxime), and *Lachnospiraceae* (for both antibiotics), as could
534 be observed over axis 1, which explains >85% of the variation. The use of multiple doses
535 of cefotaxime or of vancomycin produced distinct correlation patterns. For three

cefotaxime doses, the genes 16S rRNA, *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$ had a co-linear variation and were correlated with the decrease during incubation of populations such as *Lachnospiraceae,* as can be seen over axis 1 that explains >70% of the variation. The variation of the genes *bla*$_{\text{OXA-A}}$ and *van*A was co-linear and negatively correlated with *Firmicutes* of the families *Christensenellaceae* and *Enterococcaceae* (Figure 4C). For three vancomycin doses, the 16S rRNA gene was positively correlated with the *Lachnospiraceae* and *Burkholderiaceae,* as can be observed over axis 1 explaining >88% of the variation. With opposite distributions over axis 2 that explains < 8% of variation were the genes *bla*$_{\text{OXA-A}}$ and *van*A, *bla*$_{\text{CTX-M}}$ and bla$_{\text{TEM}}$ with a co-linear variation. The three latter were positively correlated with *Tannerellaceae* (phylum *Bacteroidetes*) and the *Enterococcaceae*, *Acidaminococcaceae* and *Christensenellaceae* (phylum *Firmicutes*) (Figure 4 D).

This study aimed at assessing if ARGs harbored by exogenous wastewater ARB in fecal material and if aerobiosis or antibiotics could influence their survival. The rationale was that variations of exogenous bacteria could be due to their fitness or due to the influence of the fecal microbiota rearrangements, or both. Previous studies have shown that enterobacteria spiked in animal feces can maintain viability for up to three months in ambient air (Scott *et al.*, 2006; Segura *et al.,* 2018; Sinton *et al.,* 2007; Walters and Field 2009). In the present study, it was used a period of 7 days, considering this would be an acceptable time period for transient intestinal colonization with wastewater ARB. The PCA and CCA results suggest that exogenous bacteria survival or proliferation is unpaired by the autochthonous fecal microbiota, mainly *Firmicutes* and *Bacteroidetes* families. However, the assayed exogenous bacteria could not be eliminated until 7 days and their genes could be detected in FMAs incubated for 30 days (data not shown).

560 Overall these results suggest that: i) the fate of ARGs is mainly determined by the fitness
561 of the host bacteria; ii) in spite of the ARGs host decay, the overall decrease of the fecal
562 bacterial population (e.g. due to adverse conditions or antibiotherapy) may lead to
563 apparent increases of ARGs prevalence, and iii) even if ARGs host cells lose viability
564 they may protect the ARGs, as long as cells integrity is maintained. The role of the
565 competitor native fecal microbiota is unquestionable, and it may be influenced by both
566 diversity and abundance. Although it is difficult to estimate the likelihood of transmission
567 of wastewater-ARB to humans, the fact that these may be able to survive the competition
568 by native microbiota is a topic that should not be neglected.
569

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581

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733

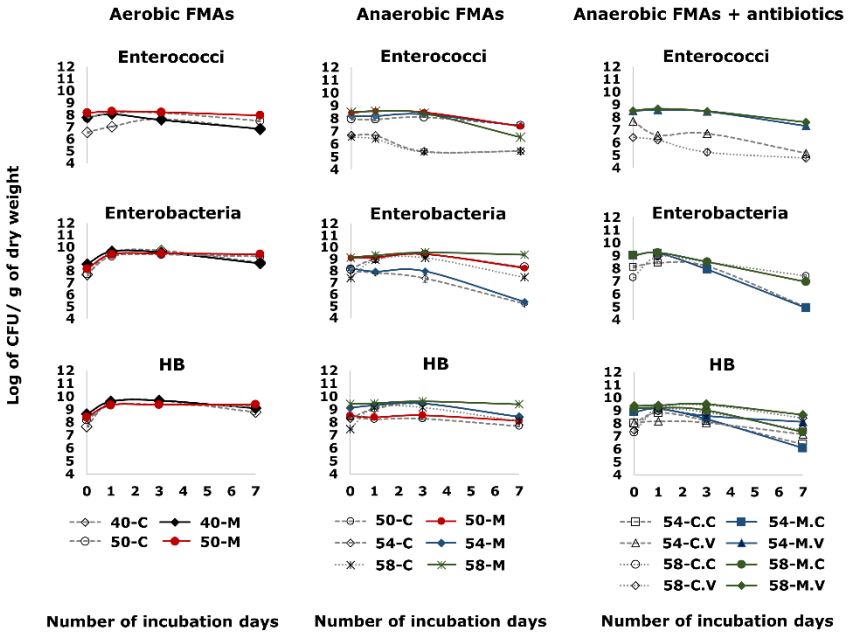

Figure 1. Colony forming units (CFUs) enumeration per gram of dry weight of stool samples, non-inoculated (C, dashed grey lines with empty symbols) or ARB-inoculated (M, solid colorful lines with full symbols) for FMAs incubated under aerobic (column on the left hand side) and anaerobic (column in the middle) conditions, and in the presence of antibiotics (column on the right hand side). FMA54 were performed in the presence of a single-dose of cefotaxime (54-M.C, blue squares) or vancomycin (54-M.V, blue triangles), while FMA58 were conducted in the presence of multiple-doses of cefotaxime (58-M.C, green circles) or vancomycin (58-M.V, green rhombus). CFUs were enumerated on m-Enterococcus, m-FC and LA medium for enterococci, enterobacteria and heterotrophic bacteria (HB) counts, respectively. The CFU values are the average of triplicates with the standard deviation. Note: At T0 of FMA-54 and FMA-58, the log CFUs on antibiotic supplemented culture media per gram of feces dry weight of the ARB-inoculated FMAs was 9.03 ± 0.11 and 9.13 ± 0.14 (54 and 58) on mFC agar with cefotaxime and, 8.55 ± 0.06 and 8.47 ± 0.55 (54 and 58) on m-Enterococcus agar with vancomycin, being below the detection limit in non-inoculated controls. At time 7 days of FMA-54 and FMA-58, the log CFUs on antibiotic supplemented culture media per gram of feces dry weight of the ARB-inoculated FMAs was 6.38 ± 0.07 and 8.06 ± 0.12 (54 and 58) on mFC agar with cefotaxime and, 7.40 ± 0.07 and 6.58 ± 0.08 (54 and 58) on m-Enterococcus agar with vancomycin, being below the detection limit in non-inoculated controls.

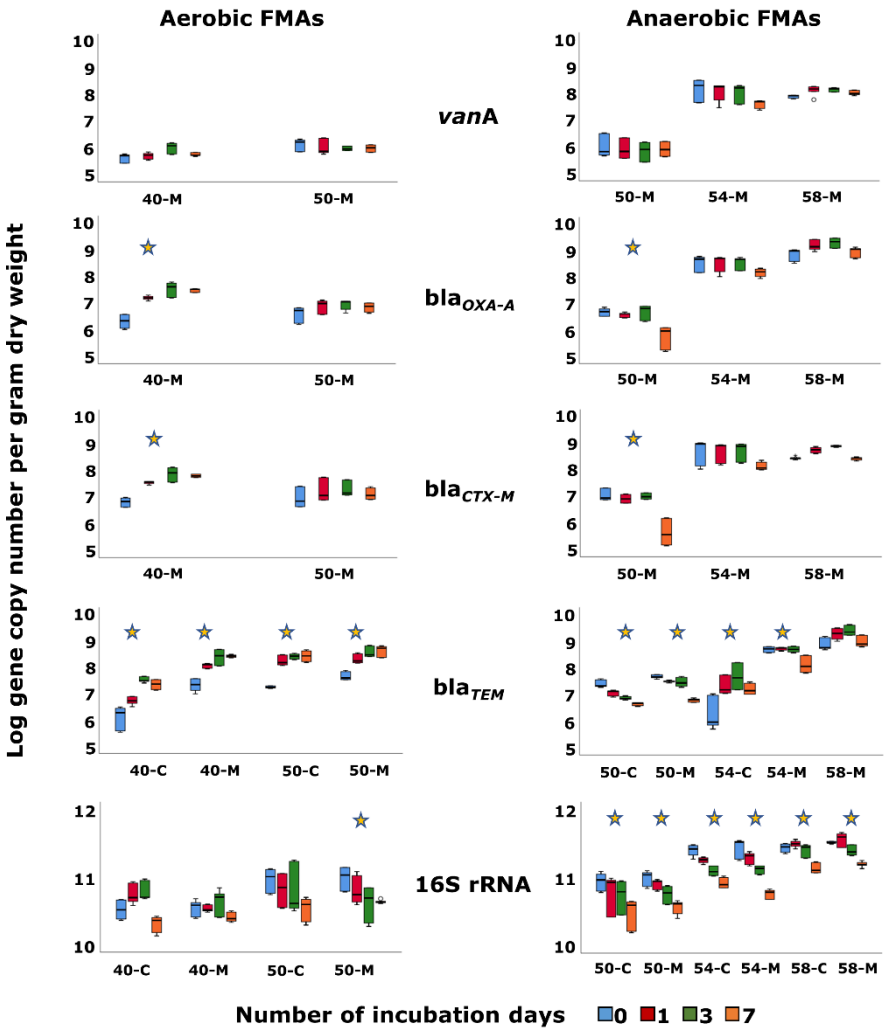

Figure 2. Variation of 16S rRNA and antibiotic resistance genes over time. The abundance of the genes (*van*A, bla$_{OXA-A}$, bla$_{CTX-M}$, bla$_{TEM}$, and 16S rRNA) per g of dry weight of stool, of non-inoculated (C) and ARB-inoculated (M) FMAs under aerobic (column on the left side) and anaerobic (column on the right) conditions is shown. The variation of each gene along time among each FMA is indicated by colored boxplot graphs (blue, red, green and orange corresponding to 0, 1, 3 and 7 days, respectively). The FMAs 40-C, 40-M, 50-C and 50-M were performed aerobically. The FMAs 50-C, 50-M, 54-C, 54-M, 58-C and 58-M were performed in anaerobic condition. With exception of the gene bla$_{TEM}$, the ARGs *bla*$_{CTX}$, *bla*$_{OXA-A}$ and *van*A were not detected in the non-inoculated microcosms. However, the gene bla$_{TEM}$ was not detected in the non-inoculated microcosms 58-C. Genes abundance are the average values of all FMAs replicates with the standard deviation. Stars indicate statistically significant variation ($p<0.01$) of the genes abundance between T0 and T7 for each FMA.

In cell-free DNA supplemented FMAs, FMA44 and FMA48, the spiked genes (in log copy number per gram of stool dry weight) were 5.92 ± 0.09 for $bla_{\text{CTX-M}}$, 5.99 ± 0.43 for $bla_{\text{OXA-A}}$, and 4.02 ± 0.13 for *van*A and 9.24 ± 0.30 for bla_{TEM}, a value that might be overestimated, given the natural occurrence of this gene in the non-inoculated microcosms. After one day of incubation the $bla_{\text{CTX-M}}$, $bla_{\text{OXA-A}}$, and *van*A genes were below the limit of quantification.

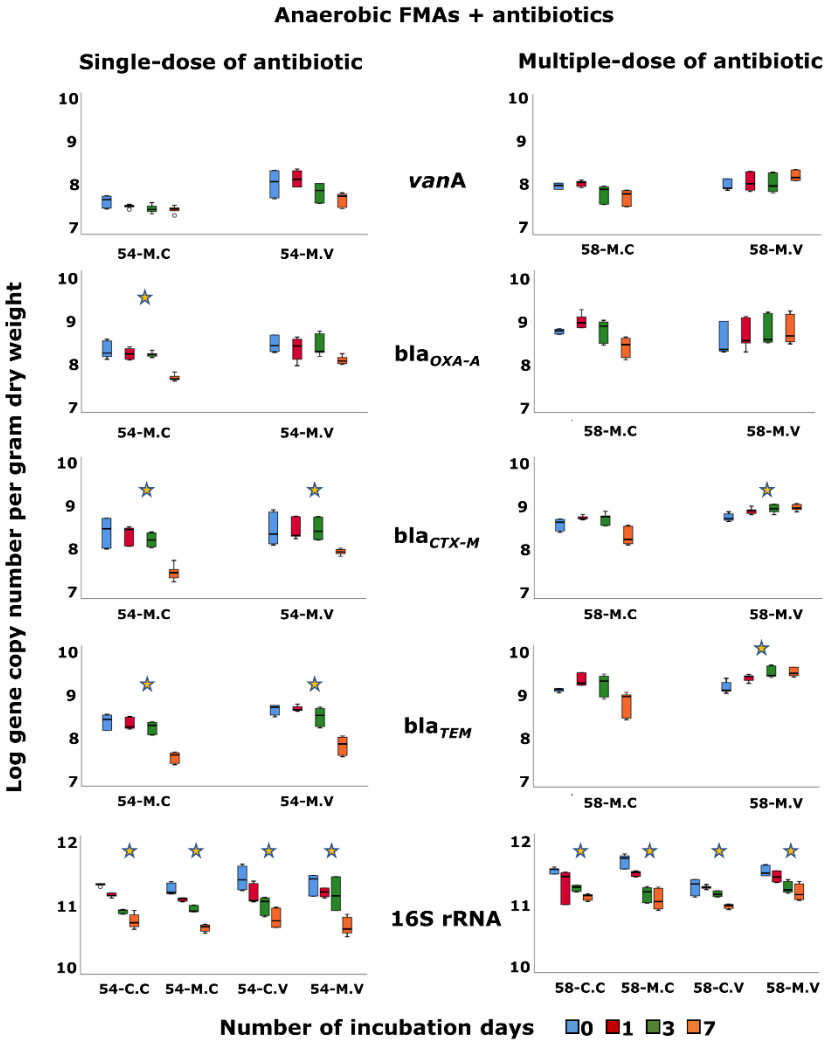

Figure 3. Effect of antibiotic on the variation of 16S rRNA and antibiotic resistance genes along the time. The abundance of the genes (*van*A, bla_{OXA-A}, bla_{CTX-M}, bla_{TEM} and 16S rRNA) per g of dry weight of stool, of non-inoculated (C) and ARB-inoculated (M) FMAs under anaerobic conditions in the presence of antibiotics is shown. The variation of each gene along time for each FMA is indicated by colored boxplot graphs (blue, red, green and orange corresponding to 0, 1, 3 and 7 days, respectively). FMAs were performed in the presence of a single-dose of cefotaxime (54-C.C, 54-M.) or vancomycin (54-C.V, 54-M.V) or, in the presence of multiple-doses of cefotaxime (58-C.C, 58-M.C) or vancomycin (58-C.V, 58-M.V). The ARGs bla_{TEM} , bla_{CTX}, bla_{OXA-A} and *van*A were not detected in the non-inoculated microcosms. Genes abundance are the average values of all FMAs replicates with the standard deviation. Stars indicated statistically significant variation ($p<0.01$) of genes abundance between T0 and T7 for each FMA.

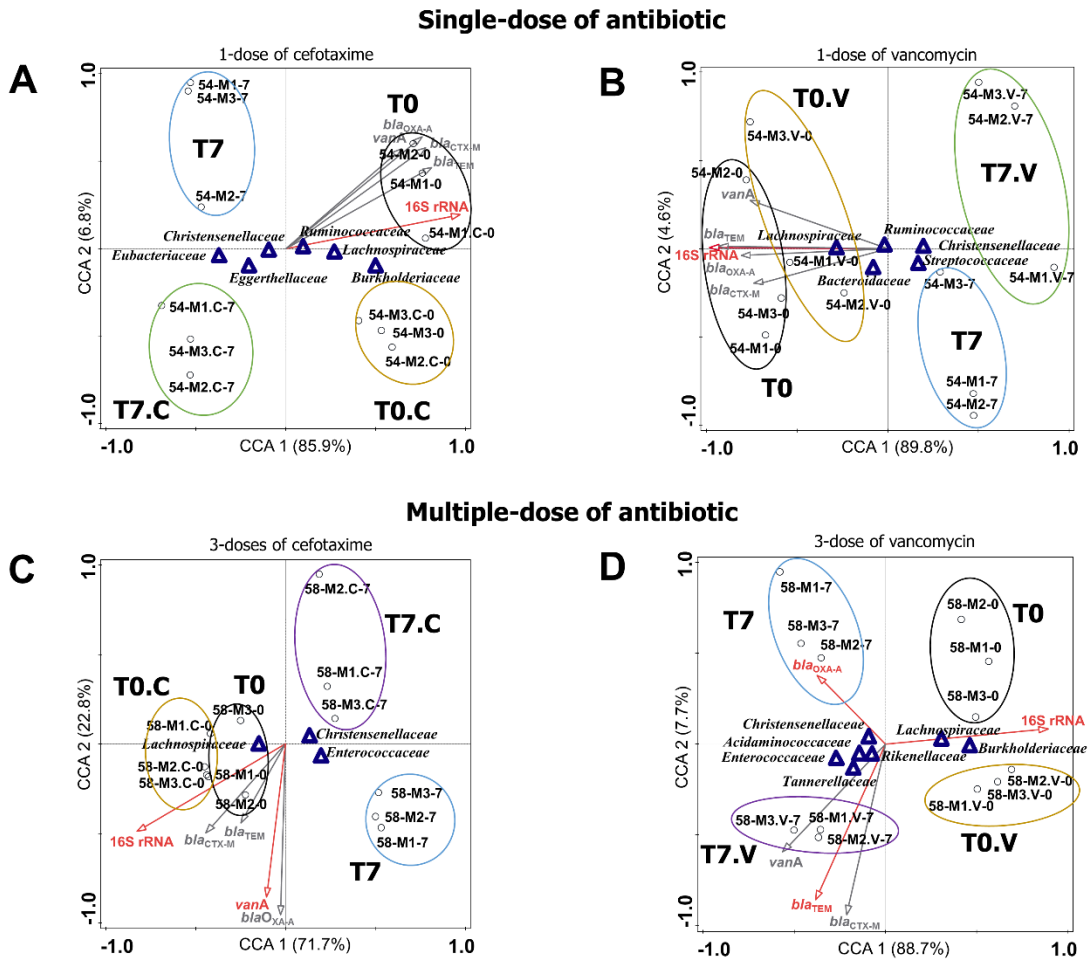

Figure 4. Canonical Correspondence Analysis (CCA) of bacterial families (with relative abundance > 1%, and with the highest fit values, > 0.90) of the ARB-inoculated assays in the presence of a single-dose of cefotaxime (**A**) or vancomycin (**B**) or, in the presence of a multiple-dose of cefotaxime (**C**) or vancomycin (**D**). All the CCA represent microcosms at the beginning (T0) and after seven days of incubation (T7) without antibiotic (black circle and light blue circle, respectively), T0 and T7 in the presence of a single-dose of cefotaxime (T0.C, yellow circle; T7.C, green circle) or vancomycin (T0.V, yellow circle; T7.V, green circle) and, T0 and T7 in the presence of a multiple-dose of cefotaxime (T0.C, yellow circle; T7.C, purple circle) or vancomycin (T0.V, yellow circle; T7.V, purple circle). The red arrows show the significant explanatory variables ($p<0.05$) while grey arrows represent the explanatory variables with no significant correlation.

Table 1. Composition and conditions of the different microcosm assays. Each FMA comprised 24 vials (12 non-inoculated - C and 12 inoculated - M).

Experiment	Inoculum				Conditions used for each assay				16S rRNA based microbiome analysis
	ARB (cell density OD_{610})		Free-DNA		Oxygen availability		Antibiotic presence		
FMA	*E. coli* A2FCC14 cell density (approx. CFU mL^{-1})	*Enterococcus* sp. H1EV10 cell density (approx. CFU mL^{-1})	*E. coli* A2FCC14 (µg)	*Enterococcus* sp. H1EV10 (µg)	Aerobic	Anaerobic	Cefotaxime (4 mg L^{-1})	Vancomycin (16 mg L^{-1})	
40-C	-	-	-	-	+	-	-	-	T0; T7
40-M	10^7	10^6	-	-	+	-	-	-	T0; T7
44-C	-	-	-	-	+	-	-	-	T0; T7
44-M	-	-	2.0	1.5	+	-	-	-	T0; T7
48-C	-	-	-	-	+	-	-	-	
48-M	10^7	10^6	-	-	+	-	-	-	
48-M	-	-	2.0	1.5	+	-	-	-	
50-C	-	-	-	-	+	-	-	-	T0; T7
50-M	10^7	10^6	-	-	+	-	-	-	T0; T7
50-C	-	-	-	-	-	+	-	-	T7
50-M	10^7	10^6	-	-	-	+	-	-	T0; T7
54-C	-	-	-	-	-	+	-	-	T0; T7
54-M	10^7	10^6	-	-	-	+	-	-	T0; T7
54-C.C	-	-	-	-	-	+	+ (1 dose)	-	T7
54-M.C	10^7	10^6	-	-	-	+	+ (1 dose)	-	T0; T7
54-C.V	-	-	-	-	-	+	-	+ (1 dose)	T7
54-M.V	10^7	10^6	-	-	-	+	-	+ (1 dose)	T0; T7
58-C	-	-	-	-	-	+	-	-	T0; T7
58-M	10^7	10^6	-	-	-	+	-	-	T0; T7
58-C.C	-	-	-	-	-	+	+ (3 doses)	-	T7
58-M.C	10^7	10^6	-	-	-	+	+ (3 doses)	-	T0; T7
58-C.V	-	-	-	-	-	+	-	+ (3 doses)	T7
58-M.V	10^7	10^6	-	-	-	+	-	+ (3 doses)	T0; T7

C, control - non-inoculated assay; C.C, control spiked with cefotaxime; C.V, control spiked with vancomycin. M, ARB-inoculated assay; M.C, ARB-inoculated assay spiked with cefotaxime; M.V, ARB-inoculated assay spiked with vancomycin.
T0, beginning; T7, seven days of incubation.

Table 2. Microcosm effect in fecal microcosm assays (FMAs) based on a single healthy donor aged 40, 44, 50, 54 and 58 months. Control, non-inoculated (C), and test, ARB-inoculated (M), FMAs incubated under aerobic (40-C, 40-M; 44-C, 44-M; 50-C, 50-M) or anaerobic (50-C, 50-M; 54-C, 54-M; 58-C, 58-M) conditions. Phyla with relative abundance equal or below 2% are designated as other phyla. The values correspond to the ratio of relative abundance (number of reads/total number of reads) of each replicate at T7 of C or M per the average values of the relative abundance at the respective T0 of C or M. The values are expressed as the average of triplicates ± the standard deviation.

FMAs	**Condition**	**Phylum relative abundance at T7/ phylum relative abundance at T0**						**Family relative abundance at T7/ family relative abundance at T0**	
		Bacteroidetes	*Actinobacteria*	*Verrucomicrobia*	*Firmicutes*	*Proteobacteria*	Other phyla (≤ 2%)	*Enterobacteriaceae*	*Enterococcaceae*
40-C	Aerobic	0.85±0.01*	1.02±0.02	0.80±0.08	0.78±0.01*	7.53±0.44*	24.94±0.71	51.90±9.43*	n.d.
40-M		0.90±0.02*	1.10±0.06	1.47±0.26	0.82±0.04*	4.81±0.59*	2.81±0.69	20.08±4.26*	0.69±0.14
44-C		1.06±0.03	1.77 ±0.05*	1.43±0.08*	0.65±0.01*	1.62±0.10*	3.12±0.74	3.05±0.14*	n.d.
44-M		1.12±0.05*	1.54±0.19*	1.41±0.07*	0.62±0.03*	1.62±0.04*	3.69±0.29	2.86±0.19*	0.26±0.45
50-C		0.79±0.05	1.13±0.06	0.64±0.07	0.99±0.09	4.83±1.02*	1.70±0.29	27.59±6.48*	n.d.
50-M		0.86±0.03	1.18±0.03	0.62±0.13	0.90±0.01	4.95±0.16*	2.18±0.46	20.72±0.38*	4.75±0.17*
50-C	Anaerobic	0.94±0.02	1.39±0.07	0.43±0.09	0.98±0.00	0.39±0.01*	2.16±0.08	0.36±0.08*	n.d.
50-M		0.93±0.09	1.44±0.19	0.47±0.13	0.96±0.01	0.33±0.05*	1.00±0.19	0.31±0.02	6.31±0.18*
54-C		0.91±0.03	1.59±0.14*	0.65±0.08*	0.83±0.04*	2.18±0.25*	1.40±0.44	28.80±3.51*	n.d.
54-M		0.98±0.03	1.32±0.13*	0.83±0.18	0.89±0.03*	0.70±0.10*	2.86±0.71	1.34±0.23	1.14±0.15
58-C		1.09±0.02	1.24±0.02*	1.28±0.18	0.81±0.01*	0.96±0.21	1.53±0.18	48.37±17.22*	n.d.
58-M		1.06±0.06	1.31±0.13	1.09±0.12	0.74±0.04*	2.46±0.43*	1.78±0.38	6.56±1.33*	3.91±0.45*

n.d., not detected both at T0 and T7.

*; statistically significant variation ($p<0.01$) between T0 and T7 for each FMA

Table 3. Antibiotic effect on non-inoculated (C) and ARB-inoculated (M) FMAs exposed to the addition of a single- (54-C.C, 54-M.C, 54-C.V, and 54-M.V) or a multiple-dose (58-C.C, 58-M.C, 58-C.V, and 58-M.V) of antibiotic. Legend: 54-C or 54-M and 58-C or 58-M, non-inoculated (C) or ARB-inoculated (M) FMA without addition of antibiotics; 54-C.C or 54-M.C and 58-C.C or 58-M.C, FMAs with addition of a single- or a multiple-dose, respectively, of cefotaxime (4 mg L^{-1}); 54-C.V or 54-M.V and 58-C.V or 58-M.V, FMA with addition of a single- or a multiple-dose, respectively, of vancomycin (16 mg L^{-1}). Phyla with relative abundance equal or below 2% were designated as other phyla. The values correspond to the ratios of the relative abundance of each replicate at T7 of C or M in the presence of antibiotic and, the average values of the relative abundance at T7 of C or M without the addition of antibiotics. The values are expressed as the average of triplicates ± the standard deviation.

FMAs	**Conditions**	**Phylum relative abundance with antibiotic/ phylum relative abundance without antibiotic**						**Family relative abundance with antibiotic/ family relative abundance without antibiotic**	
		Bacteroidetes	*Actinobacteria*	*Verrucomicrobia*	*Firmicutes*	*Proteobacteria*	Other phyla (≤ 2%)	*Enterobacteriaceae*	*Enterococcaceae*
54-C	Single-dose of antibiotic cefotaxime (C) or vancomycin (V)	1.00±0.03	1.00±0.09	1.00±0.13	1.00±0.04	1.00±0.11	1.00±0.31	1.00±0.12	n.d.
54-M		1.00±0.03	1.00±0.10	1.00±0.21	1.00±0.03	1.00±0.14	1.00±0.25	1.00±0.17	1.00±0.13
54-C.C		1.08±0.08	0.86±0.09	1.20±0.14	1.08±0.02	0.26±0.04*	1.25±0.21	0.12±0.03*	n.d.
54-M.C		1.00±0.09	0.86±0.12	1.26±0.30	1.09±0.04	0.39±0.02*	1.07±0.09	0.14±0.03*	1.04±0.15
54-C.V		1.02±0.10	0.90±0.12	1.28±0.22	1.10±0.01	0.19±0.02*	1.10±0.23	0.06±0.01*	n.d.
54-M.V		0.96±0.04	0.98±0.07	1.14±0.20	1.06±0.04	0.45±0.16	1.01±0.25	0.23±0.17	1.88±0.79
58-C	Multiple-dose of antibiotic cefotaxime (C) or vancomycin (V)	1.00±0.02	1.00±0.02	1.00±0.14	1.00±0.01	1.00±0.22	1.00±0.12	1.00±0.36	n.d.
58-M		1.00±0.06	1.00±0.10	1.00±0.11	1.00±0.05	1.00±0.17	1.00±0.22	1.00±0.20	1.00±0.12
58-C.C		0.80±0.07	1.19±0.08	0.73±0.12	1.01±0.00	1.30±0.39	1.52±0.48	1.46±0.47	n.d.
58-M.C		0.89±0.13	1.19±0.16	0.91±0.23	1.09±0.01	0.18±0.03*	1.27±0.33	0.13±0.03*	0.78±0.05
58-C.V		0.80±0.38	1.16±0.51	0.71±0.32	0.98±0.44	2.76±1.22*	1.07±0.51	3.47±0.85*	n.d.
58-M.V		1.01±0.05	1.02±0.04	0.94±0.17	1.05±0.06	0.68±0.14	0.63±0.15	0.63±0.15	1.18±0.17

n.d., not detected at T7.

*, statistically significant difference ($p<0.01$) between the FMAs with the addition of antibiotic versus no addition of antibiotic, for each FMA at time 7 day