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Commensal *E. coli* rapidly transfer antibiotic resistance genes to human intestinal microbiota in the Mucosal Simulator of the Human Intestinal Microbial Ecosystem (M-SHIME)

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ABSTRACT

Food-producing animals are indicated as a reservoir of antibiotic resistance genes and a potential vector for transmission of plasmid-encoded antibiotic resistance genes by conjugation to the human intestinal microbiota. In this study, transfer of an antibiotic resistance plasmid from a commensal *E. coli* originating from a broiler chicken towards the human intestinal microbiota was assessed by using a Mucosal Simulator of the Human Intestinal Microbial Ecosystem (M-SHIME). This *in vitro* model mimics the human intestinal ecosystem and received a single dose of 10^9 *E. coli* MB6212, which harbors a plasmid known to confer resistance towards several antibiotics including tetracycline, sulfamethoxazole and cefotaxime. Since the degree of stress imposed by stomach pH and bile acids vary with the consumed meal size, the effect of meal size on *E. coli* donor survival and on plasmid transfer towards lumen and mucosal coliforms and anaerobes was determined.

The administered commensal *E. coli* strain survived stomach acid and bile salt stress and was able to grow in the colon environment during the timeframe of the experiment (72 h). Transfer of antibiotic resistance was observed rapidly since cultivable transconjugant coliforms and anaerobes were already detected in the lumen and mucosa after 2 h in the simulated proximal colon. The presence of the resistance plasmid in the transconjugants was confirmed by PCR. Differences in meal size and adapted digestion had neither a detectable impact on antibiotic resistance transfer, nor on the survival of the *E. coli* donor strain, nor on short chain fatty acid profiles. The median number of resistant indigenous coliforms in the lumen of the inoculated colon vessels was 5.00×10^5 cfu/ml [min - max: 3.47×10^4–3.70×10^8 cfu/ml], and on the mucosa 1.44×10^7 cfu/g [min-max: 4.00×10^3–4.00×10^8 cfu/g]. Exact quantification of the anaerobic transconjugants was difficult, as (intrinsic) resistant anaerobic background microbiota were present. QPCR data supported the observation of plasmid transfer in the simulated colon. Moreover, inoculation of *E. coli* MB6212 had no significant impact on the microbial diversity in the lumen as determined by 16 S ribosomal gene based next generation sequencing on lumen samples.

This study demonstrates that a commensal, antibiotic resistant *E. coli* strain present in food can transfer its antibiotic resistance plasmid relatively quickly to intestinal microbiota in the M-SHIME. The spread and persistence of antibiotic resistance genes and resistant bacteria in our intestinal system is an alarming scenario which might present clinical challenges, since it implies a potential reservoir for dissemination to pathogenic bacteria.

1. Introduction

The spread and proliferation of antibiotic resistance genes to pathogens is an emerging problem, threatening effective treatment of bacterial infections (World Health Organization, 2017). Although the occurrence of antibiotic resistance is a natural phenomenon, the misuse

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and overuse of antibiotics in livestock is considered as one of the drivers of the increasing resistance development (Munk et al., 2018). Aside from the thoroughly explored antibiotic resistance in bacterial zoonotic pathogens, a large mainly unexplored pool of resistant, commensal bacteria exists in the environment and the food production chain. These commensal bacteria are considered harmless but can be a reservoir of mobile genetic elements (such as plasmids and transposons) carrying antibiotic resistance genes that may be disseminated horizontally to pathogens. It is known that these mobile elements can be highly promiscuous as transfer can occur between different species, genera and even between Gram positives and –negatives (Courvalin, 1994; Doucet-Populaire et al., 1992; Ochman et al., 2000). Several studies indicate the potential spread of antibiotic resistance between livestock, food supply and humans (reviewed by Muloi et al. (2018)). Although the directionality of the resistance transfer could not always be elucidated, livestock and food supply may act as a potential vector for transmission of plasmid encoded antibiotic resistance genes by conjugation to the human intestinal microbiota.

Surveillance data of food at retail level to assess the exposure of the consumer to antibiotic resistant bacteria *via* food are rare and often limited to raw meat samples (European Food Safety Authority, 2018). A recent study reported a median antibiotic resistant bacteria prevalence level of > 50% on retail food samples (Jans et al., 2018). There is a growing concern that antibiotic resistant bacteria present on food can transfer their resistance genes to the inherent gut microbiota of the consumer. The human gastrointestinal tract is one of the most densely populated ecosystems known, with an estimated number of 10^{13}–10^{14} microbial inhabitants and is regarded as a hotspot for gene transfer (Salyers et al., 2004). Quantitative data and risk assessment studies about the effect of human exposure to antibiotic resistant bacteria through consumption of contaminated food or food derived products and resistance transfer and persistence in our gut are scarce. The first *in situ* indication of horizontal antibiotic resistance transfer from an ingested strain towards an intestinal acceptor strain is traced back to Smith in 1969. In his pioneering study, a human volunteer ingested a non-resistant *E. coli* acceptor strain, followed by various concentrations of antibiotic resistant *E. coli* donor strains. Although resistance transfer was observed to a limited extent, the resistant acceptor strains did not persist for long in the gut. Whereas this study only visualized *E. coli* intraspecies transfer in the human gut, using an experimental design that nowadays would face ethical constraints, other studies confirmed the transfer of antibiotic resistance genes in the gut of gnotobiotic mice (Gruzza et al., 1993; Moubareck et al., 2003) and rats (Jacobsen et al., 2007) or in *in vitro* reactor conditions mimicking rumen (Toomey et al., 2009), porcine caecum (Peeters et al., 2017) and human colon (Haug et al., 2011; Smet et al., 2011). Inoculation of a resistant donor strain in a colon reactor, as performed in the published *in vitro* studies, however, bypasses the challenging effects of the stomach (low pH) and ileum (bile salts and pancreatic enzymes) on the donor strain (Smith, 1969).

In the current study, the transfer of an antibiotic resistance plasmid from a foodborne commensal *E. coli* strain towards the human intestinal microbiota was investigated. We used the M-SHIME® (Mucosal Simulator of the Human Intestinal Microbial Ecosystem), which is a dynamic *in vitro* gut model that mimics the entire gastrointestinal tract, incorporating stomach, small intestine and colon. The microbial community in these gastrointestinal compartments has a highly similar structure and function to the one in the human gastrointestinal system. Moreover, the M-SHIME contains mucin covered microcosms, which enables studying the adherent community on the mucus layers, besides the luminal community, in the simulated colon.

The objectives of this study were (i) to evaluate whether a commensal antibiotic resistant *E. coli* strain can survive in the Mucosal Simulator of the Human Intestinal Microbial Ecosystem (M-SHIME) after consumption, (ii) to assess if and how quickly antibiotic resistance transfer occurs from the consumed resistant *E. coli* strain to the intestinal microbiota and to (iii) reveal if the size of the meal containing the resistant *E. coli* has an influence on its survival and plasmid transfer, since the degree of stress by stomach pH and bile acids vary along the consumed meal size.

2. Materials and methods

2.1. Characteristics and cultivation E. coli plasmid donor strain

A commensal resistant *Escherichia coli* strain MB5876, previously isolated from a broiler caecum (Lambrecht et al., 2018) was selected for this study. This strain contains a 117 kb-plasmid, p5876 (GenBank accession number MK070495), harboring several antibiotic resistance genes, among which those conferring resistance to cefotaxime, tetracycline and sulfamethoxazole.

To track plasmid transfer in the M-SHIME, *E. coli* MB6212, a lactose-negative derivative of MB5876 was used, since this enabled to differentiate between the plasmid donor (*E. coli*, MB6212) and transconjugants (*i.e.* indigenous bacteria which accepted the plasmid). The former grew as white colonies on MacConkey plates with selected antibiotics, whereas the latter formed red colonies. Selection of lactose-negative mutants was performed as described by Smet et al. (2011). The lac-mutation did not hamper conjugative transfer, since similar *in vitro* (37 °C, LB-broth, 24 h) transfer ratios were obtained compared to the wild-type strain. In addition, lactose-negative MB6212 appeared as pink colonies on RAPID'*E. coli* Agar (Biorad, California, USA), while wild-type strain (MB5876) and other innate *E. coli* formed purple colonies, favoring separate enumeration.

The MB6212 strain was stored at −80 °C and grown overnight in Tryptone Soy Broth (Thermo Fisher Scientific, Waltham, USA) at 37 °C, washed and diluted to 10^8 CFU/ml in ¼ Ringers solution (Thermo Fisher Scientific) before inoculation in the SHIME stomach.

2.2. Mucosal Simulator of the Human Intestinal Microbial Ecosystem (M-SHIME®) setup

The SHIME® reactor setup (registered name from Ghent University and ProDigest, Belgium) was adapted to run four SHIME systems in parallel (2 treatment conditions and 2 corresponding controls), each consisting of a succession of a stomach, small intestine and proximal colon vessel (Fig. 1). The proximal colon was characterized by a pH of 5.6–6.0, a volume of 500 ml and a retention time of 20 h. Each colon vessel had an integrated mucosal compartment to study both luminal and mucosa-associated bacteria.

A fresh fecal sample from a single human volunteer (female, 28 years) with a Western diet and who had no history of antibiotic treatment five years prior to the sample collection was collected according to standard procedures. The sample was homogenized, aliquoted, flash frozen with liquid nitrogen and stored at −80 °C until use. Use of fecal samples from human origin for *in vitro* research was approved by the Ghent University hospital ethical committee under Belgian registration number BE670201214538.

At the beginning of the M-SHIME experiment, the colon vessels were inoculated with 25 ml of a 20% (*w/v*) fecal slurry (prepared as described by De Boever et al. (2000) and 475 ml nutritional medium (Prodigest, NM002A containing 1.2 g/l arabinogalactan, 2 g/l pectin, 0.5 g/l xylan, 0.4 g/l glucose, 3 g/l yeast extract, 1 g/l special pepton, 2 g/l mucin, 0.5 g/l l-cystein-HCl and 4 g/l starch). A mucosal environment was created by adding 80 mucin type II-agar covered microcosms (AnoxKaldnes K1 carrier; AnoxKaldnes AB, Lund, Sweden) per colon vessel (Vermeiren et al., 2012). Every two days, 50% of the microcosms was replaced by new ones to simulate the natural renewal of the mucus layer. During the start-up period, three times a day, 140 ml acidified nutritional medium (pH 2, HCl) and 60 ml pancreatic juice (6 g/l oxgall (BD, Erembodegem, Belgium), 0.9 g/l porcine pancreatin (Sigma Aldrich, Overijse, Belgium), 12.5 g/l $NaHCO_3$ (Carl Roth, Karlsruhe, Germany) were added to the stomach and small

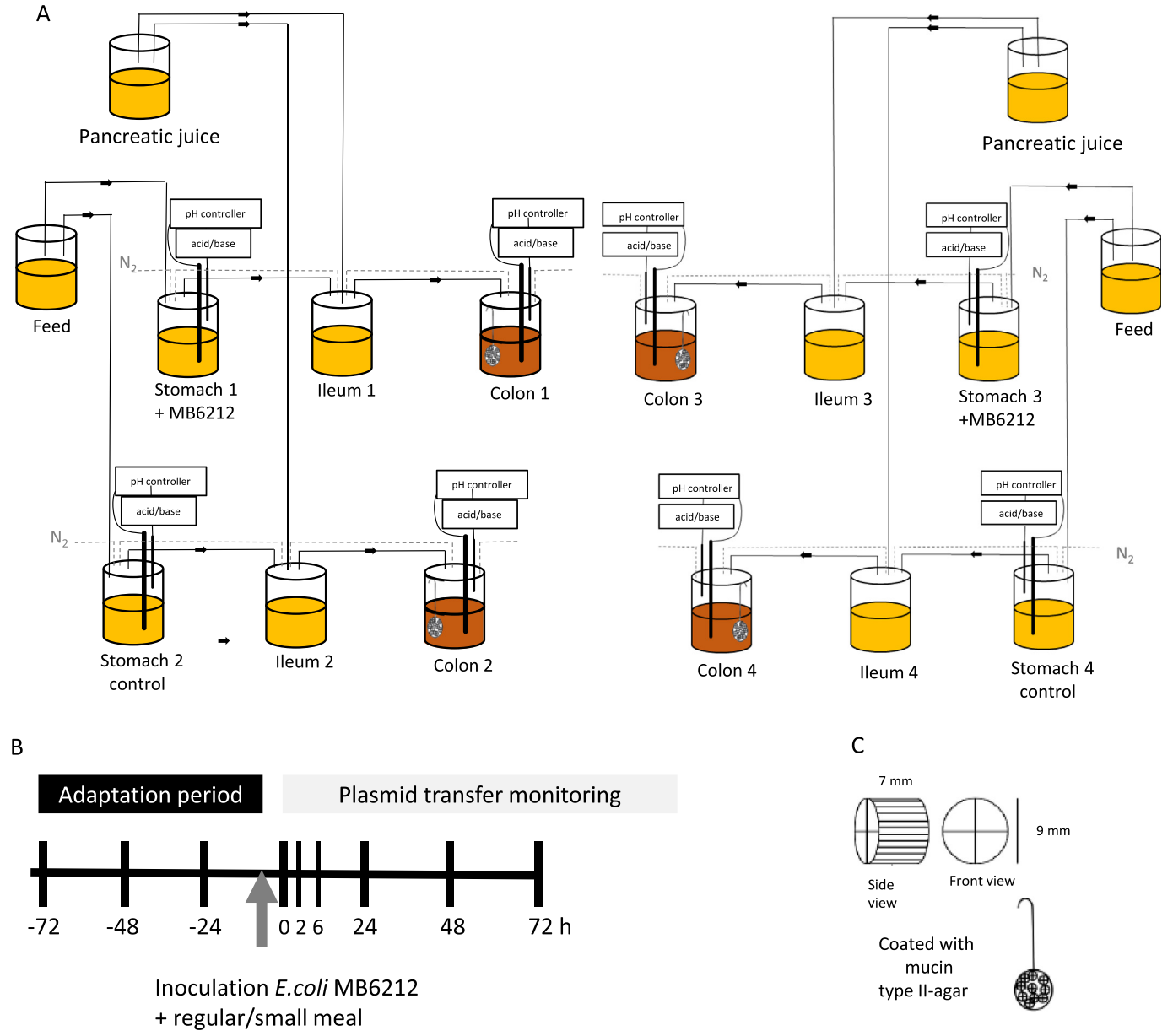

Fig. 1. M-SHIME setup
A: Reactor vessel setup
B: Time frame of the experiment. 0 h: time point at which *E. coli* MB6212 enters the colon vessel
C: Mucin microcosms.

intestinal compartments, respectively (Van den Abbeele et al., 2013). This start-up period enabled the microbial community to adapt to the nutritional and physicochemical conditions in the colon vessels.

After 3 days of adaptation, the stomach vessels were inoculated with 10 ml 10^8 cfu/ml *E. coli* MB6212, while the control stomach vessels were inoculated with 1/4 Ringers solution. At the same time either 14 ml (small meal) or 140 ml nutritional medium (normal meal) was added to the stomach vessels. For the small meal size, the pH of the corresponding stomach vessels was maintained at pH 2, whereas for the normal meal size, the pH was gradually lowered from 5 to 2, mimicking *in vivo* conditions.

After the MB6212 inoculation, all stomach vessels were continued to be fed with 140 ml nutritional medium and 60 ml pancreatic juice three-times a day for 72 h. During the whole experiment, anaerobic conditions were maintained by daily flushing with N_2.

Sampling of the simulated lumen and mucosa was performed at regular time points (Fig. 1). Lumen samples (10 ml) were aliquoted for DNA extraction, SCFA analysis and bacterial counts. Mucosal samples for bacterial counts were taken by removing the microcosms covered in mucin agar from the colon vessels, washing them three times in phosphate buffer (0.1 M, pH 6.8, containing 1 g l^{-1} sodium thioglycolate as reducing agent, Sigma Aldrich, Overijse, Belgium) to remove loosely attached cells and harvesting the mucin. Approximately 0.2 g mucin was added to 800 µl Ringer, vortexed vigorously and used for bacterial plating.

The M-SHIME setup (Fig. 1) was repeated four times over time, starting from frozen aliquots (snap frozen in liquid N_2) from a single fecal sample of one human volunteer to test reproducibility. In a last setup, all stomach vessels (2 treatments and 2 controls) were fed with a regular meal upon inoculation with MB6212. Hence, in total, five replicate runs were performed with a regular meal and three replicate runs with a small meal.

2.3. Bacterial counts

Lumen and mucosa samples were serially diluted in 1/4 Ringers solution and plated on various selective plates. Total coliforms were enumerated on MacConkey agar (nr3, Oxoid) and total anaerobic bacteria on Reinforced Clostridial Agar (RCA, Oxoid). Resistant bacteria (transconjugants and the MB6212 donor strain) were counted on MacConkey and RCA with cefotaxime (0.25 μg/ml) in combination with either tetracycline (32 μg/ml) or sulfamethoxazole (256 μg/ml). The antibiotic concentrations were chosen based on preliminary experiments in order to (I) surpass coliform Epidemiological Cut Off (ECOFF) values, (ii) be lower than the MIC of the donor strain, and (iii) inhibit or reduce growth of resistant background microbiota if present in the fecal material of the human donor. Indigenous *E. coli* and the MB6212 strain were enumerated separately on RAPID'E.coli (Biorad). All plates were incubated at 37 °C for 24 h. Reinforced Clostridial Agar plates were incubated in anaerobic jars with anaerogen sachets (Oxoid).

In general, no significant differences in resistant bacterial counts were observed between the agar plates with cefotaxime-tetracycline and those with cefotaxime-sulfamethoxazole ($p > 0.05$, clustered Wilcoxon rank test). Only graphs related to MacConkey agar/RCA plates with cefotaxime-sulfamethoxazole are displayed in the results section.

Transconjugants on MacConkey agar with antibiotics were differentiated from MB6212 by their red morphology. To verify that their resistance was caused by p5876 and not due to intrinsic resistance or a spontaneous mutation, a PCR assay with primers targeting a fragment of a specific and unique region on p5876 (116,086–117,066 bp, primerBLAST search June 2017) was performed. Ten to 15 resistant colonies per colon vessel were randomly selected, lysed with 0.25% SDS, 0.1 M NaOH (17 min, 90 °C) and used in the PCR assay. In total 150 lysates from resistant coliforms were analyzed by PCR.

On the RCA plates with antibiotics, anaerobic transconjugants and the MB6212 donor could not be discriminated. To get a rough estimation of the number of true transconjugants among the resistant colonies, 10 RCA colonies per colon vessel were picked up and streaked on RAPID'E.coli (pink colonies after incubation originate from MB6212) and a fresh RCA plate. RCA colonies correlating to non-pink colonies on RAPID'*E. coli* (n = 100) were lysed with lysostaphine (Sigma, 1 mg/ml, 10 min. 37 °C) followed by proteinase K treatment (Promega, 2.5 mg/ml, 10 min 60 °C and 5 min 100 °C) and analyzed in the PCR assay described above.

PCRs were carried out in a total volume of 25 μl containing 1 × buffer II (Invitrogen), 1.5 μM $MgCl_2$, 1.5 U Ampli Taq DNA polymerase (Applied Biosystems, California, US), 200 μM dNTPs, 0.8 μM of each primer ($FW_{start116897bp}$: 5′-GATTTGAAGCGCACTCAGCG-3′ and $RV_{start117087bp}$: 5′-TAAGTTGGCAGCATCACCT CGG-3′) and 1 μl crude cell lysate. The reaction parameters were as follows: 3 min denaturation at 95 °C, followed by 30 cycles of 30 s. at 95 °C, 30 s at 56 °C and 1 min. at 68 °C and a final extension at 68 °C for 5 min. The amplicons (191 bp) were detected using the QIAxcel capillary electrophoresis system (Qiaxcel DNA Screening Cartridge, Method AM320, QIAGEN GmbH, Hilden, Germany).

2.4. SCFA analysis

Luminal SCFA levels were measured using capillary gas chromatography (GC-2014 gas chromatograph (Shimadzu, Hertogenbosch, the Netherlands) coupled to a flame ionization detector after diethyl ether extraction, as described by Andersen et al. (2014). The total SCFA is the sum of acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, caproate and isocaproate.

2.5. Statistical analysis of bacterial counts and SCFA concentrations

All statistical analyses were performed in R (version 3.5.1). Linear mixed models were used for modelling the absolute and relative bacterial counts of cultivable resistant coliforms, anaerobes and MB6212 in lumen and mucosa. Coliforms, anaerobes and MB6212 counts were tested separately, as were lumen and mucosa data. Sampling time points, total coliforms and anaerobes, MB6212 inoculation, meal size and SCFA concentrations were included as potential fixed effects. Reactor run was considered as random factor to include reactor variability. Similar models were fitted for the concentrations (nmol/L) of acetate, propionate, butyrate and total SCFA. The normality of the residuals of each model was confirmed by a QQ-plot and a histogram. When significant effects were present (ANOVA, $p < 0.05$), a posthoc analysis using Tukey's test was performed. To compare lumen and mucosa transferratios (#resistant/total bacteria) at specific timepoints a clustered Wilcoxon rank sum test (clustrank v0.6-2) was used.

2.6. DNA extraction

Total DNA was extracted from the pellet of 1 ml lumen samples or 0.20 g mucin according to a protocol adapted from Geirnaert et al. (2015). Cells were lysed with 1 ml lysis buffer (100 mM Tris/HCl pH 8.0, 100 mM EDTA pH 8, 100 mM NaCl, 1% (m/v) polyvinylpyrrolidone and 2% (m/v) sodium dodecyl sulphate) and 200 mg glass beads (0.11 mm) in a BioLyzer (MoBio, Carlsbad, USA) for 5 min, 2000 rpm. Following removal of glass beads by centrifugation (5 min, 15,000 g), DNA was extracted from supernatant using a phenol:-chloroform:isoamyl alcohol (25:24:1, pH 7) extraction. The DNA was precipitated at −20 °C with 1 volume of ice-cold isopropyl alcohol and 0.1 volume of 3 M sodium acetate for at least 1 h. After removal of isopropyl alcohol by centrifugation (30 min, maximum speed), the DNA pellet was dried, resuspended in 100 μL 1 × TE (10 mM Tris, 1 mM EDTA) buffer and immediately stored at −20 °C until further analysis. A quality control PCR was performed using Taq DNA Polymerase with the Fermentas PCR Kit according to the manufacturers' specification (Thermo Fisher Scientific, Waltham, MA, USA).

2.7. Quantification of p5876 by qPCR

Quantitative PCR was used to quantify (i) total p5876 in lumen and mucine samples, independent of its host (MB6212 donor or transconjugants) and (ii) total 16S rRNA gene as a proxy for bacterial load. QPCR analysis was performed on a Lightcycler 480 Real-time PCR system (Roche) using SYBR Green technology. For each DNA extract, a 1000-fold dilution was made and analyzed in duplicate. Dilutions were made to exclude inhibitor effects. The 25 μl qPCR mix consisted of SsoAdvanced Universal Inhibitor-Tolerant SYBR Green supermix (Biorad), 5 μl diluted DNA extract and plasmid specific primers (600 nM, FW 5′-GGCTGAGAAAGCCCAGTAAGG-3′, RV: 5′-TAAGTTGG CAGCATCACCTCG-3′) or eubacterial 16S primers (100 nM, Bac338_F 5′-ACTCCTACGGGAGGCAGCAG-3′ and Bac518_R 5′-TTACCGCGGCTG CTGG-3′, (Ovreås et al., 1997)). The PCR program consisted of an activation step of 3 min at 98 °C, followed by 30 cycles of 10 s at 98 °C and 30 s at 60 °C. Within each run, standard curves were constructed using 10-fold dilution series of a 16S plasmid construct (pIDTSMART-AMP, IDT, Coralville, IA, USA) or p5876 gblock (1237 bp artificial sequence consisting of regions [1–932 bp] and [116804–117,108 bp] of p5876) for quantification purposes. The qPCR efficiencies for p5876 and total 16S rRNA gene were [97.9–108.3%] and [97.5–102.4%] respectively. Melting curve analysis was conducted over a range of 60 °C to 95 °C, in steps of 0.3 °C s^{-1} to assess specificity of the amplification products.

2.8. 16S amplicon sequencing and bioinformatics analysis

The lumen microbial community at 0 and 72 h from three M-SHIME runs was assessed using Illumina next generation 16S rRNA gene amplicon sequencing. DNA extraction was performed as reported by Geirnaert et al. (2015). Samples were sent out to LGC Genomics for

library preparation and sequencing on an Illumina MiSeq platform. The 341F-785R primerpair, derived from Klindworth et al. (2013), with a slight modification to the reverse primer by introducing another degenerated position (K) was used to amplify the 16S rRNA V3-V4 region.

The mother software package (v1.39.5, Schloss et al. (2009)) was used to process the Illumina data. Forward and reverse reads were assembled into contigs and ambiguous contigs or those with divergent lengths were removed. The number of unique sequences was determined and these were aligned to the Mothur-reconstructed SILVA Seed alignment (v123). Sequences not aligning within the region targeted by the primer set or sequences with homopolymer stretches with a length higher than 12 were removed. Sequences were pre-clustered together within a distance of 1 nucleotide per 100 nucleotides. These cleaned-up and preclustered sequences were checked for chimera's (with Uchime) (Edgar et al., 2011). The sequences were classified using SILVA version v128 and a naive Bayesian classifier (Wang's algorithm). All sequences that were classified as Eukaryota, Archaea, Chloroplasts and Mitochondria were removed. If sequences could not be classified at all (even not at (super)Kingdom level) they were removed. The operational taxonomic units (OTUs) were clustered with an average linkage and at the 97% sequence identity. Singletons (reads occurring only once in all samples) were considered noise and were omitted during data analysis.

The microbial community structure within lumen samples was assessed by calculating Hill numbers (H_0, H_1 and H_2) on absolute counts by Phyloseq (v1.24.2). A Wilcoxon (Signed) Rank Sum test was used to detect significant differences in Hill numbers between reactor runs, sampling time points and MB6212 inoculated *vs*. controls vessels.

Differences in microbial community composition between samples were explored using RC(M) ordination with reactor run as confounder (fit Row-Column association Models, RCM package v0.1.0, Center For Statistics UGent). Significant differences were identified by means of Permutational Multivariate Analysis of Variance (PERMANOVA) using the adonis function (vegan v.2.5-2). Multivariate homogeneity of dispersion (variance) was calculated with the betadisper function (vegan).

The raw fastq files that were used to create the OTU table, which served as a basis for the microbial community analysis, have been deposited in NCBI database (Accession number PRJNA493203).

3. Results and discussion

The transfer of antibiotic resistance genes from consumed bacteria to our gut microbiota is a risk that has been poorly addressed (European Food Safety Authority, 2008, 2018). Therefore, in the current study an M-SHIME system was fed with a nutritional medium containing 10^9 antibiotic resistant *E. coli* MB6212, harboring the transferable resistance plasmid p5876, and the emergence of resistant gut microbiota was monitored over time. To mimic different degrees of digestive stress in the gastric and intestinal compartments, the antibiotic resistant *E. coli* MB6212 was dosed with either a regular or small meal. Digestive conditions were adapted accordingly. Upon entering the proximal colon, the resistant *E. coli* (max. 2×10^6/ml) encounter *c.* 10^{11} intestinal compartment bacteria/ml which may act as potential plasmid acceptors. This led to an estimated initial donor:acceptor ratio of 1:10^5, not taking into account increasing or decreasing *E. coli* donor concentrations due to gastrointestinal passage, plasmid host range and plasmid incompatibilities (Gregory et al., 2008).

Transfer of the resistance plasmid p5876 was quickly observed, as after 2 h residing in the simulated colon, resistant, indigenous coliforms could be detected in lumen and mucosa (Fig. 2). Meal size, sampling time point and site (lumen or mucosa) had no significant impact on the absolute number of resistant coliforms (data not shown), nor on the resistance ratio (*i.e.* the number of non-MB6212 resistant coliforms/total coliforms, Fig. 2). Overall, the median number of resistant indigenous coliforms in the lumen of the inoculated colon vessels was 5.00×10^5 cfu/ml [min - max: 3.47×10^4–3.70×10^8 cfu/ml], and on the mucosa 1.44×10^7 cfu/g [min-max: 4.00×10^3–4.00×10^8 cfu/g]. 96% (n = 150) of the selected resistant colonies tested positive for the presence of p5876 by PCR. Resistant coliforms, may have obtained their plasmid either (i) directly from MB6212 by an initial plasmid transfer event, (ii) by second level plasmid transfer from transconjugants or (iii) by vertical transfer during growth. Remarkably, from 24 h onwards, resistant coliforms appeared in the control vessels, albeit at significantly ($p = 0.0004$) lower concentrations than in the MB6212 inoculated vessels. This is most likely caused by outgrowth of intrinsic resistant microbiota or microbiota harboring other mobile resistance elements – already present in M-SHIME inoculum - to levels above the detection limit. The presence of p5876 in the resistant colonies originating from the control vessels could not be detected by PCR, vouching this hypothesis. Although spontaneous mutations could also lead to antibiotic resistance (Martinez and Baquero, 2000) this event is less plausible, since spontaneous bacterial mutants usually arise at low frequencies (10^{-6} to 10^{-8} (Briales et al., 2011)) and there was no antibiotic pressure in the M-SHIME.

Plasmid p5876 is an IncFII plasmid: as IncF plasmids are assumed to be narrow host range plasmids (Dang et al., 2016) transfer is mainly expected to occur to related taxa, justifying the plating on MacConkey agar. However, parallel plating on RCA was performed as well, as the predominant microbiota in the human gut is strictly anaerobic. On RCA plates, resistant anaerobes (up to 5.9 log at 2 h and 6.2 log at 72 h) where detected both in control and MB6212-inoculated vessels, hampering the exact quantification of plasmid transfer. Only 23% (n = 100) of the selected putative transconjugants on RAPID'*E. coli* harbored p5876 as determined by PCR, which suggests the presence of resistant anaerobic background microbiota. Resistance towards cefotaxime, tetracycline or sulfamethoxazole among several anaerobes including enterococci (Hollenbeck and Rice, 2012), bifidobacteria (Masco et al., 2006) and lactic acid bacteria (Karapetkov et al., 2011) has been reported before. The mechanisms underlying these resistances are either intrinsic to the species or acquired through mutations in intrinsic genes or mobile elements encoding resistance determinants.

Cultivable transconjugants were detected in both simulated lumen and mucosa samples. The mucin layer in the M-SHIME is colonized by a biofilm community which enhances cell-to-cell contact and hence can improve the initial efficiency of conjugative transfer of some plasmids, but on the other hand, can limit further plasmid spread into the community (Stalder and Top, 2016). Differences in plasmid transfer efficiency in lumen and mucosa samples could not be deducted from the current data, as this would require more extensive mucin sampling at short time intervals and considering transfer towards non-cultivable bacteria. The current data prove that a resistance plasmid of a commensal *E. coli* can be transferred quickly to colon bacteria, which highlights the dynamic potential of gut microbiota genomes.

Meal size had no detectable impact on the occurrence of resistant coliforms and strict anaerobes ($p > 0.05$). Bacteria consumed with a small meal will encounter a prompter pH drop in the stomach, and lower bile salt concentrations in the ileum compared to those consumed with a regular meal. *E. coli* MB6212 survived gastro-intestinal conditions irrespective of the meal size and persisted in the proximal colon in the presence of established colon microbiota. Viable *E. coli* MB6212 donors could be recovered from both lumen and mucosa (Fig. 3). The ability of *E. coli* to grow and survive in mucus has been shown to be critical for *in vivo* intestinal colonization (Chang et al., 2004). Moreover, growth tests performed by Poulsen et al. (1995) on streptomycin treated mice inoculated with one *E. coli* strain indicated that growth only occurred on mucus while luminal *E. coli* were static. These results contradict our findings, where growth in both lumen and mucosa seems to take place. This could be attributed to strain and host differences. Nonetheless, the observed increase in concentration may also be caused by recolonization from lumen to mucus and *vice versa*.

The copy number of p5876, irrespective of its host, was quantified in the simulated lumen and mucosa samples by means of qPCR. As

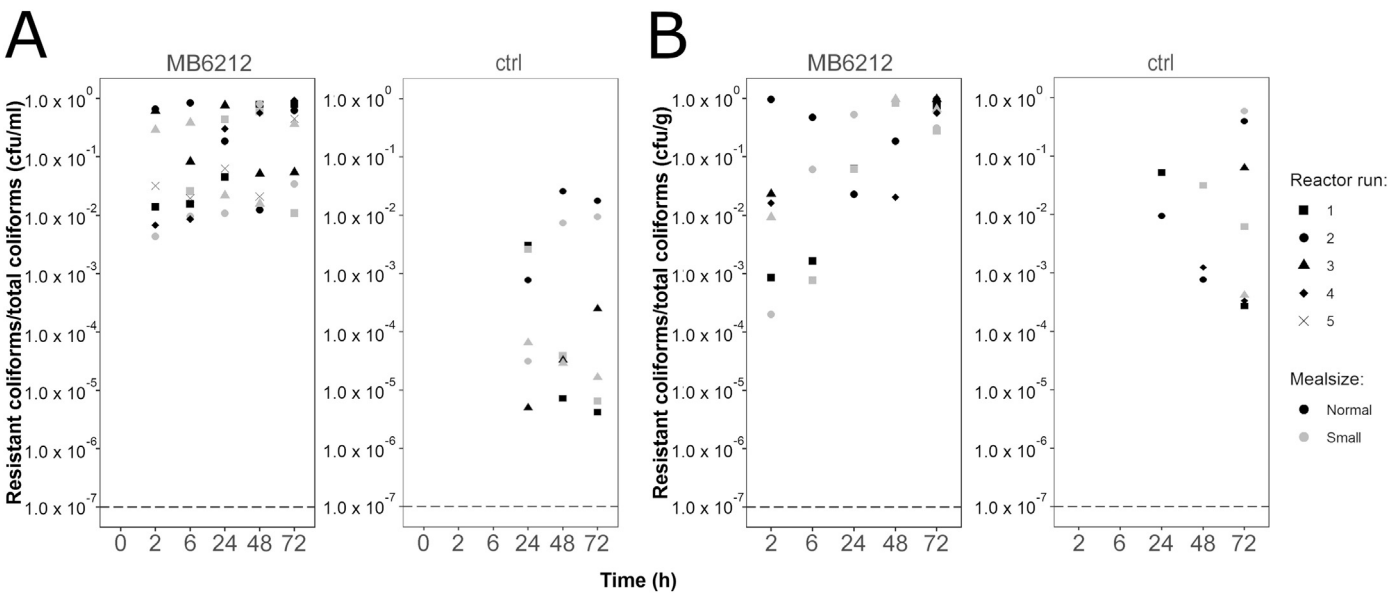

Fig. 2. Emergence of resistant coliforms in the lumen (A) and mucosa (B) in the M-SHIME over time.
Stomach vessels of the M-SHIME were inoculated with 10^9 cfu *E. coli* harboring a conjugative antibiotic resistance plasmid (MB6212) or Ringer (control setup, ctrl), during consumption of a normal (140 ml) or small meal (14 ml). *E. coli* reached the proximal colon vessel at time point 0h. Resistant non-MB6212 coliforms in the proximal colon vessels were enumerated by selective plating.
Time: residence time in the colon, dashed line: detection limit. Five replicate M-SHIME reactor runs were performed.
Reactor run 4 and 5: small meal size not tested, and mucus samples not taken at 6 h and 24 h.
Meal size, sampling time point and site (lumen or mucosa) had no significant impact on the resistance ratio (linear mixed effects model with Tukey's post-hoc test, $p < 0.05$)

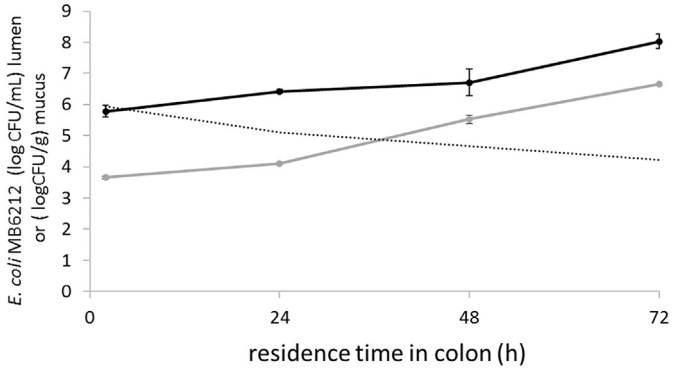

Fig. 3. Persistence of *E. coli* MB6212 in lumen (black line) and mucosa (grey line) of the proximal colon (M-SHIME) *E. coli* MB6212 (10^9 cfu) were inoculated in the stomach vessels and reached the proximal colon at 0 h. Dashed line: theoretical MB6212 wash-out from reactor vessel, when no growth. (mean ± SE, n = 3, normal meal size samples).

expected, at -72 h and 0 h (stomach inoculation time point), no p5876 was detected in the colon lumen and mucus samples. In the lumen samples a significant increase in p5876/16S ratio was observed from 2 h ($5.78 \times 10^{-3} \pm 2.16 \times 10^{-3}$, mean ± standard error) to 72 h ($6.90 \times 10^{-2} \pm 1.95 \times 10^{-2}$). This increase wasn't detected for the resistant coliforms and anaerobes count data, suggesting that uncultivable bacteria also harbor the plasmid. No significant p5876/16S increase was observed for mucus samples over time, at 72 h the ratio was $2.04 \times 10^{-2} \pm 1.21 \times 10^{-2}$ (mean ± standard error). The qPCR results support the observation of plasmid transfer in the proximal colon and suggest that the real number of transconjugants is likely to be higher than the ones detected on the agar plates. This fits the expectations, since the majority of the gut microbiota is considered uncultivable (Browne et al., 2016).

16S ribosomal gene based amplicon sequencing was applied to lumen samples at time points 0 and 72 h from control and MB6212-inoculated vessels to detect potential shifts in community composition. Illumina sequencing of the V3-V4 16S rRNA gene fragment revealed that all lumen samples were dominated by Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria, phyla known to predominate in gut microbial communities (Hugon et al., 2015). At genus level, *Lachnoclostridium*, *Escherichia/Shigella*, *Veillonella*, *Bifidobacterium*, some unclassified *Enterobacteriaceae* and *Bacteroides*, were most abundant (Fig. 4). Collectively, these genera comprised at least 96.7% of the abundance in each sample. Applying the BLAST algorithm with type material on the consensus sequence of the two OTU's clustered under unclassified Enterobacteriacea led to following best hits: OTU1 (Identity: 100%, Query coverage: 100): *Leclercia adecarboxylata*, *Pantoea agglomerans*, *Enterobacter cloacae*, *Enterobacter ludwigii* and *Enterobacter adecarboxylata*, OTU2 (Identity: 99%, Query coverage: 100): *Klebsiella pneumoniae*, *Klebsiella quasipneumoniae* and *Phytobacter diazotrophicus*. Inoculation of *E. coli* MB6212, and reactor run had no significant impact on the microbial diversity in the lumen as determined by Hill numbers. An RCM ordination of the luminal microbial community at OTU level indicated that sampling time point is the main factor determining the grouping of samples (Fig. 5), which was confirmed by PERMANOVA ($p = 0.007$). *Bacteroides* (OTU6 and OTU 12) and *Escherichia-Shigella* (OTU2) were more abundant at 72 h, whereas *Veilonella* (OTU5) and *Lachnoclostridium* (OTU17) were more abundant at 0 h. The main conclusion to be drawn from the 16S amplicon data is that adding 10^9 resistant *E. coli* MB6212 did not disrupt the bacterial community composition in the simulated proximal colon.

Short Chain Fatty Acids (SCFA) serve as markers for bacterial metabolic activity and have been shown to exert health benefits (den Besten et al., 2013). In this study, SCFA concentrations were used to assess steady-state after the adaptation period and monitor the effect of MB6212 inoculation. Reactor run, inoculation with MB6212, meal size during inoculation and sampling time point had no detectable ($p > 0.05$) effect on the total SCFA production, nor on the molar ratio of acetate, propionate and butyrate (data not shown). The total overall SCFA concentration was 74 ± 1.91 nM (mean ± standard error,

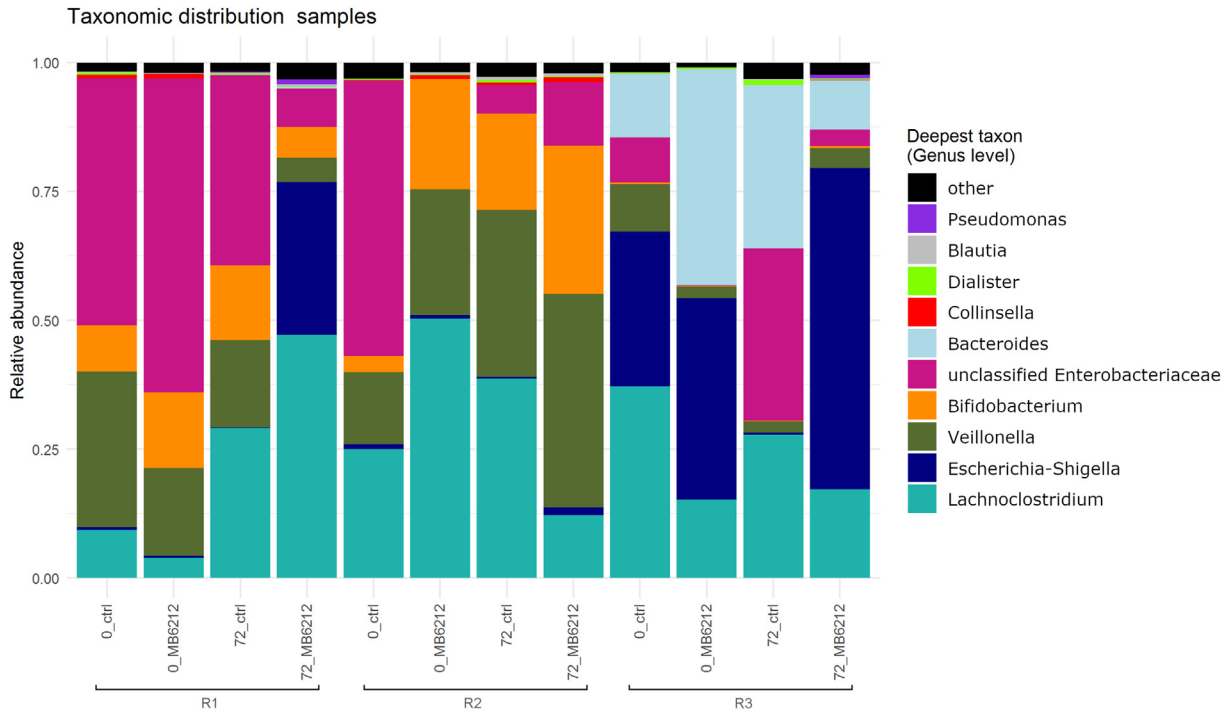

Fig. 4. Relative abundance of the 10 most abundant genera of the luminal microbial community based on Illumina sequencing of the V3-V4 16S rRNA gene fragment 0 & 72 indicate sampling time points (h) of the M-SHIME proximal colon vessels with (MB6212) or without (ctrl) resistant *E. coli* MB6212. Time point 0 h: just before *E. coli* MB6212 starts entering the colon vessel. R1-R3: independent, replicate M-SHIME run.

n = 96), which lays within the range of the *in vivo* proximal colon concentrations 70–140 mM (Topping and Clifton, 2001). SCFA production is host and diet dependent, which might explain why the mean acetate:propionate:butyrate ratio of 83:13:4, deviates from the expected 60:20:20 to 70:15:15 ratio (Brussow and Parkinson, 2014; Hamer et al., 2009). Nevertheless, this ratio occurred independently in each M-SHIME reactor run and remained stable during the experimental timeframe. These results indicate that there were no measurable changes in bacterial metabolism during the M-SHIME reactor run, not even after inoculation of the non-indigenous commensal *E. coli* MB6212.

In conclusion, this proof of concept study demonstrates that an animal-derived, commensal, antibiotic resistant *E. coli* strain can transfer its resistance genes relatively quickly to intestinal microbiota in the M-SHIME, even without clear selective pressure (*i.e.* without antibiotic use). Humans can be exposed to viable, commensal antibiotic resistant *E. coli* by contact with livestock or a contaminated natural environment and by inadequately cooked food or cross-contamination. A recent study reported a median antimicrobial resistance prevalence of > 50% for meat and seafood at retail level (Jans et al., 2018). However, the exact daily dose to which we are exposed is not known yet. Depoorter et al. (2012) estimated that the risk of being exposed to > 1000 cfu cephalosporin resistant *E. coli* after consumption of a prepared meal with chicken meat is 1,5%. The actual exposure risk for consumers is multifactorial and depends on consumption preferences, good hygiene practices and if the products undergo a cooking step prior to consumption (Jans et al., 2018).

In this study, the M-SHIME was fed with 10^9 cfu commensal *E. coli* MB6212, leading to an initial proximal colon concentration of max. 2×10^6 cfu/ml, representing a worst-case scenario. By running several independent M-SHIMEs with aliquoted fecal material from a single human donor, we demonstrated that the plasmid transfer is a

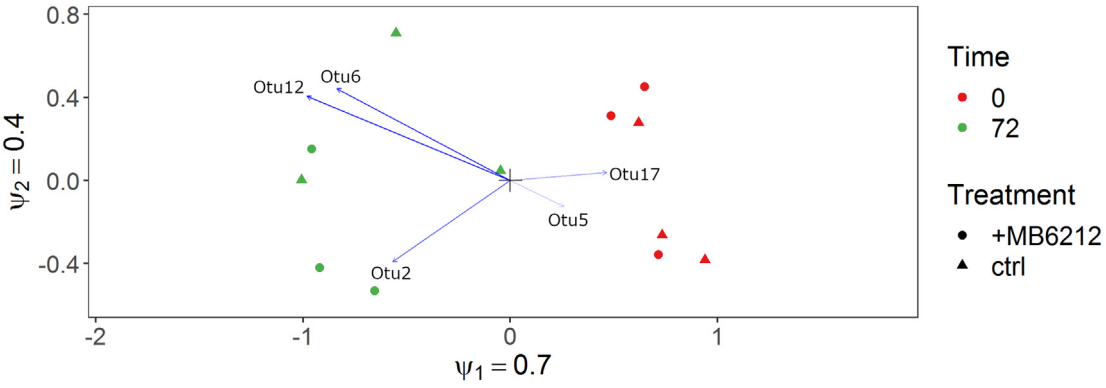

Fig. 5. RCM ordination describing the lumen microbial community composition at OTU level, as determined by Illumina sequencing of the V3-V4 16S rRNA gene fragment

Proximal colon vessels with (+MB6212) or without (ctrl) antibiotic resistant *E. coli* MB6212. Samples were taken at 0 h and after 72 h. Time point 0 h: just before MB6212 starts entering the colon vessel.

OTU6 and OTU12 (Bacteroides), OTU2 (Escherichia-Shigella), OTU5 (Veilonella) and OTU17 (Lachnoclostridium).

reproducible event. Whether MB6212 and the transconjugants are able to persist in the colon for a long time (> 72 h) and whether they preserve their plasmid cannot be deduced from the current data. Like in many other bacteria, the resistance plasmid, p5876, contains a toxin-antitoxin system, suggesting that the plasmid can be stably retained in bacteria for a long time. Transmission of antibiotic resistance plasmids from food related commensals towards indigenous gut microbiota and possibly ingested pathogens is an alarming process which requires further attention. Prevention and/or decrease of transfer may be a possible but still underexplored tool to limit the emergence and spread of multi-resistant bacteria.

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Declaration of competing interest

All authors disclose no commercial associations that might create a conflict of interest in connection with this study.

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