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Lacticaseibacillus rhamnosus GG and *Saccharomyces cerevisiae boulardii* supplementation exert protective effects on human gut microbiome following antibiotic administration *in vitro*

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RESEARCH ARTICLE

Abstract

Antibiotic-induced dysbiosis of the microbial community has been associated with several gastrointestinal symptoms. The impact of repeated administration of *Lacticaseibacillus rhamnosus* GG (CNCM-I-4798) (formerly known as *Lactobacillus rhamnosus* GG), *Saccharomyces cerevisiae boulardii* (CNCM-I-1079) and their combination (associated in Smebiocta/Smectaflora Protect®) in supporting recovery of gut microbiota functionality and composition during and following amoxicillin:clavulanic acid administration was evaluated *in vitro*. Antibiotic dosage negatively affected SCFA production, coinciding with detrimental effects on *Bacteroidetes*, *Firmicutes* and *Bifidobacterium* spp. in the simulated proximal colon, while *Akkermansia muciniphila* was significantly reduced in the distal colon. *L. rhamnosus* GG and *S. boulardii* were able to thrive in both colon regions upon dosing, with *S. boulardii* even showing protective effects on the survival of *L. rhamnosus* GG during antibiotic administration. The impact of the probiotic strains on microbiome recovery revealed that supplementation with *L. rhamnosus* GG and/or *S. boulardii* resulted in a stimulating effect on the most abundant bacterial groups within the bacterial community of each donor. For one of the donors tested, co-dosing of *L. rhamnosus* GG and *S. boulardii* resulted in superior short-chain fatty acid recovery accompanied by a stronger increase in abundance of *Bifidobacteriaceae*. Overall, the current study provides first evidence that combined supplementation of *L. rhamnosus* GG and *S. boulardii* might be an interesting candidate in limiting detrimental effects of amoxicillin:clavulanic acid on the human gut microbiome, though further studies are warranted to confirm these findings.

Keywords: *Bifidobacterium*, yeast, antibiotic-associated diarrhoea, amoxicillin, clavulanic acid

1. Introduction

Although there is growing concern on aspects related to their use, antibiotics are still widely consumed. In 2017, the mean consumption of antibiotics for systemic use in Europe was 21.8 defined daily doses per 1000 inhabitants per day, with penicillins being the most commonly used class of antibiotics with a total consumption rate ranging from 36 to 71% (European Center for Disease Prevention and Control, 2018). Amoxicillin is the most widespread used penicillin-class antibiotic and is generally combined with clavulanic acid that functions as β-lactamase inhibitor, thereby avoiding amoxicillin inactivation by β-lactamase-secreting bacteria (White *et al.*, 2004). While penicillin-class antibiotics are effective in eradicating many pathogens causing bacterial infections, they also adversely affect the indigenous gut microbial community (Brötz-Oesterhelt and Brunner, 2008). The resulting antibiotic-induced colonic imbalance has been linked with several gastrointestinal symptoms. A common disturbance includes antibiotic-associated diarrhea (AAD), which is most often caused by amoxicillin therapy (McFarland, 2008). There is a need to develop strategies that limit antibiotic-induced microbial imbalance, thereby potentially reducing

the incidence of antibiotic-associated gastrointestinal symptoms.

Probiotics are characterised as live microorganisms that confer a health benefit on the host when administered in adequate amounts, probably by supporting maintenance of a balanced gut microbiome (Hill *et al.*, 2014). While probiotic intake has been linked with a variety of physiological effects, several studies reported a potential role of probiotics as preventive and/or therapeutic agent against AAD (Agamennone *et al.*, 2018). *Lacticaseibacillus rhamnosus* GG (formerly known as *Lactobacillus rhamnosus* GG) is one of the most well-documented probiotic strains for human application and several studies reported significantly reduced prevalence of AAD upon supplementation of this probiotic strain *in vivo* (Mantegazza *et al.*, 2018; Szajewska and Kolodziej, 2015; Vanderhoof *et al.*, 1999). In fact, formulations containing a minimum dose of 2×10^9 cfu *L. rhamnosus* GG has been recommended for prevention of AAD in the Netherlands (Agamennone *et al.*, 2018). Furthermore, several other health-promoting properties have been linked with *L. rhamnosus* GG including regulation of immune function (Capurso, 2019), antipathogenic activity (De Keersmaecker *et al.*, 2006; Spacova *et al.*, 2020) and prevention of atopic disease (Kalliomaki *et al.*, 2001). Another widely used probiotic with proven health-promoting effects is the yeast *Saccharomyces cerevisiae boulardii* (Lazo-Vélez *et al.*, 2018). Several human studies reported significant reductions of ADD upon intake *S. boulardii* in both children and adult patients (Kotowska *et al.*, 2005; McFarland *et al.*, 1995). However, results are conflicting, with Pozzoni *et al.* (2012) and Ehrhardt *et al.* (2016) showing absence of effect of *S. boulardii* intake on the development of AAD in elderly hospitalised patients. This strengthens the need to identify specific probiotics that could accompany specific antibiotic therapies.

Despite their clinical relevance, *in vivo* studies often fail in providing mechanistic insight in how probiotics support host health. *In vitro* approaches (Minekus *et al.*, 2014, 1999; Molly *et al.*, 1993) offer a suitable alternative as strict control of environmental factors allows evaluation of cross-talk between the indigenous microbial community and the probiotic strain(s) at the site of action. However, as intestinal models depend on human faecal samples to obtain a representative microbiome, it is important to account for inter-individual variation not only during *in vivo*, but also during *in vitro* trials (Duysburgh *et al.*, 2019a).

The main objective of the current study was to evaluate the impact of repeated doses of *L. rhamnosus* GG (CNCM-I-4798), *S. boulardii* (CNCM-I-1079) and a combination of both strains in supporting recovery of gut microbiota functionality and composition upon co-supplementation with the antibiotic amoxicillin:clavulanic acid. For this purpose, the validated Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) was used (Van de Wiele *et al.*, 2015; Van den Abbeele *et al.*, 2018), thereby considering the potential inter-individual variability in microbiome composition among human individuals.

2. Materials and methods

Chemicals and test product

All chemicals were obtained from Sigma-Aldrich (Overijse, Belgium) unless stated otherwise. Lallemand Health Solutions Inc. (Blagnac, France) provided the test strains *S. boulardii* (CNCM-I-1079) and *L. rhamnosus* GG (CNCM-I-4798), associated in Smebiocta/Smectaflora Protect®. The products were administered once a day at a daily dose of 1×10^{10} *L. rhamnosus* GG (CNCM-I-4798) and/or 4×10^9 *S.boulardii* (CNCM-I-1079). The applied dosage corresponds to *in vivo* dosages as aligned with the AAD dose recommendations (Guarner *et al.*, 2017), considering the *in vitro* survival rate of the strains during upper gastrointestinal transit (results not shown).

Simulator of the Human Intestinal Microbial Ecosystem

An adaptation of the SHIME model (ProDigest and Ghent University, Ghent, Belgium) (Molly *et al.*, 1993) was used to simulate the human gastrointestinal tract. The reactor configuration was modified to a QuadSHIME setup allowing the study of four different test conditions in parallel (Dupont *et al.*, 2019). Each arm of the QuadSHIME consisted of a succession of three reactors simulating the different regions of the gastrointestinal tract, i.e. the upper gastrointestinal tract (with subsequent simulation of the gastric and small intestinal phase), the proximal colon (PC) and the distal colon (DC), respectively (Supplementary Figure S1). While the upper gastrointestinal tract reactor is of the fill-and-draw principle, the PC and DC reactors are continuously stirred and have a constant volume and pH control. The PC was operated at pH 5.6-5.9 with a retention time of 20 h, while environmental parameters in the DC included a pH-range of 6.6-6.9 and a retention time of 32 h. In order to simulate the luminal and mucus-associated microbiota, mucin-covered microcosms were included in the PC and DC (Supplementary Figure S1) as described by Van den Abbeele *et al.* (2012). Inoculum preparation, temperature settings, feeding regime and reactor feed composition were implemented from Possemiers *et al.* (2004). To evaluate the properties of four different test conditions for two healthy human adult donors (donor A: F, 24 years; donor B: F, 25 years), two parallel QuadSHIME experiments were conducted (i.e. each donor was tested in a separate QuadSHIME setup). Informed consent was retrieved for each of the donors to use the faecal samples for the study. Upon inoculation with the faecal inoculum, a two-week stabilisation period was initiated to allow the microbiome to differentiate in the different colonic reactors.

Subsequently, baseline parameters were established during a one-week control period, after which antibiotic dosage was initiated. Amoxicillin:clavulanic acid (2:1; Toku-E, Sint-Denijs-Westrem, Belgium) was administered to the PC reactors during each feeding cycle, reaching a concentration of 150 mg/day, for a period of 5 days. This antibiotic dosage was determined in order to reach the maximum *in vivo* quantity that would reach the large intestine. Indeed, *in vivo* amoxicillin is generally dosed orally at concentrations of 1,500 mg/day (De Velde *et al.*, 2016). Out of this total daily dose, 90% is absorbed in the small intestine (Legen *et al.*, 2006), resulting in a colonic dose of 150 mg/day. This colonic dose was during the current experiments dosed to the PC reactors, spread over three feeding cycles. Furthermore, *in vivo* amoxicillin is supplied in combination with clavulanic acid, with clavulanic acid being absorbed for approximately 73% (EMEA, 2001). Generally, the administered amoxicillin:clavulanic acid ratio is 4:1 or 7:1, resulting in a ratio of 1.5:1 up to 2.6:1 in the colon when considering absorption rates of 90%:73%. Therefore, an amoxicillin:clavulanic acid ratio of 2:1 was used in this study.

In the aforementioned 5-day antibiotic period, the four arms of each QuadSHIME were operated as follows: (1) control arm (CTRL), only receiving antibiotic; (2) antibiotic + *L. rhamnosus* GG (CNCM-I-4798) once per day (LGG), (3) antibiotic + *S. boulardii* (CNCM-I-1079) once per day (SB) and (4) antibiotic + a combination of *L. rhamnosus* GG (CNCM-I-4798) and *S. boulardii* (CNCM-I-1079) once per day (LGG+SB). The probiotic strains were administered once per day (i.e. during one feeding cycle) to the PC reactor, and this approximately 2 h after the antibiotic had been dosed during that feeding cycle. Administration to the PC reactor was performed as an enteric dosing strategy was envisioned.

After cessation of antibiotic administration, a one-week treatment period followed in which each experimental arm of QuadSHIME was further supplemented with their respective probiotic test products. Finally, a wash-out period of three weeks was initiated in which each QuadSHIME was operated as during the control period to assess the recovery of the gut microbiota.

Enumeration of probiotic strains

Starting from the control period, samples were collected three times per week from the luminal environment of all colonic reactors to determine the number of cfu of the probiotic strains by spread plating. Ten-fold dilution series were prepared from these samples in phosphate buffered saline (PBS) and subsequently transferred to petri dishes containing selective agar media. Selective quantification of *L. rhamnosus* GG was performed through plating on LAMVAB agar media (Hartemink *et al.*, 1997) and plates were subsequently incubated aerobically at 37 °C for at least 72 h. Selective quantification of *S. boulardii* was performed through plating on Sabouraud agar media supplemented with chloramphenicol (50 mg/l) and plates were subsequently incubated aerobically at 30 °C for at least 36 h.

Functionality of gut microbiota

Starting from the control period, samples for determination of microbial functionality were collected three times per week from both the PC and DC. Measurement of short-chain fatty acid (SCFA) levels, including acetate, propionate, butyrate and total SCFA concentrations, was performed as reported by De Weirdt *et al.* (2010). Lactate levels were determined using a commercially available enzymatic assay kit (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions.

Community composition of gut microbiota

Starting from the control period, samples for microbiota profiling were collected once per week from the luminal environment in each colonic vessel. DNA was isolated as described by Boon *et al.* (2003), with some minor modifications as recently reported by Duysburgh *et al.* (2019b). Quantitative polymerase chain reaction (qPCR) assays for *Akkermansia muciniphila*, *Bacteroidetes* and *Firmicutes* phyla, *Lactobacillus* spp. and *Bifidobacterium* spp. were conducted using a QuantStudio 5 Real-Time PCR system (Applied Biosystems, Foster City, CA USA). Each sample was analysed in technical triplicate and outliers (more than 1 C_T difference) were removed. The qPCR for the *Firmicutes* and *Bacteroidetes* phyla were performed as described by Guo *et al.* (2008), while the qPCR for *A. muciniphila* was conducted as reported in Collado *et al.* (2007). The qPCR assay for *Lactobacillus* spp. was adopted from Furet *et al.* (2009), while the qPCR for *Bifidobacterium* spp. was performed as reported in Rinttilä *et al.* (2004). Subsequently, microbiota community composition was assessed by 16S-targeted Illumina sequencing analysis using isolated DNA originating from samples collected at the end of the control, treatment and wash-out periods. The genomic DNA extracts were diluted in DNase/RNase/protease-free water to obtain a concentration of 50 ng/µl, of which 30 µl was sent to LGC genomics GmbH (Germany) for library preparation and sequencing on an Illumina Miseq platform with v3 chemistry using primers 341F (5'-CCT ACG GGN GGC WGC AG-3') and 785Rmod (5'-GAC TAC HVG GGT ATC TAA KCC-3'), adopted from Klindworth *et al.* (2013).

Statistics

Comparison of normally distributed data of the different experimental periods on markers of microbial functionality and microbial community parameters was performed with

a Student's t-test for pairwise comparisons. Differences were considered significant if $P<0.05$. For the 16S-targeted Illumina sequencing analysis, read assembly and clean-up was largely derived from the MiSeq procedure (Kozich *et al.*, 2013; Schloss and Westcott, 2011). In brief, the Mothur software (v. 1.40.5) was used to assemble reads into contigs, perform alignment-based quality filtering (alignment to the Mothur-reconstructed SILVA SEED alignment, v. 123), remove chimeras, assign taxonomy using a naïve Bayesian classifier (Wang *et al.*, 2007) and the SILVA NR software v132, and cluster contigs into operational taxonomic units (OTUs) at 97% sequence similarity. Sequences classified as *Eukaryota*, *Archaea*, chloroplasts and mitochondria and sequences that could not be classified, even not at (super) Kingdom level, were omitted. For each OTU, representative sequences were picked as the most abundant sequence within that OTU.

3. Results

Colon-region specific colonisation of microbial community

The main phyla in the microbial community of both donors under investigation included Firmicutes, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* and a minor fraction of *Synergistetes* and *Lentisphaerae*, though the latter was only detected in microbial community of donor B (Table 1). Specific colonisation patterns were observed in the different colonic regions. *Verrucomicrobia*, *Lentisphaerae* and *Synergistetes* specifically inhabited the DC, which was also observed for several families within the *Bacteroidetes* (e.g. *Rikenellaceae*) and *Firmicutes* (e.g. *Ruminococcaceae*) phylum, while *Bifidobacteriaceae* and *Veillonellaceae* primarily colonised the PC. Further, the biofilm covering the mucin-covered microcosms contained a highly diverse microbial community. A species-specific colonisation of the lumen versus the mucus layer was observed, with *Bacteroidaceae* specifically colonising the luminal environment, while higher levels of *Lachnospiraceae* and *Ruminococcaceae* were observed in the mucus layer. Further, higher abundance of *Synergistetes* was detected in the mucosal environment, while *Verrucomicrobia* were specifically present in the lumen. Finally, strong donor-dependent differences were observed in terms of microbial community composition. While donor A was mainly characterised by high proportions of *Bifidobacteriaceae*, *Lachnospiraceae* and *Akkermansiaceae* (the latter only in the DC), donor B showed high abundance of *Veillonellaceae*. Furthermore, several bacterial families could specifically be linked to one of the donors under investigation, such as *Muribaculaceae* and *Prevotellaceae* for donor A, and *Barnesiellaceae*, *Eubacteriaceae* and *Victivallaceae* for donor B.

Pervasive effects of antibiotic administration on endogenous microbiota

Antibiotic dosage in the absence of probiotic supplementation (CTRL) resulted in reduced SCFA levels in the PC of both donors tested (Figure 1). While butyrate levels were also reduced in the DC upon antibiotic administration for both donors tested, propionate production was stimulated towards the end of the antibiotic period in this colonic area. On the other hand, acetate levels were slightly reduced in the DC of donor A, while gradually increasing levels were observed during antibiotic dosage for donor B.

In terms of community composition, as assessed by qPCR (Table 2 and Supplementary Table S1), amoxicillin:clavulanic acid dosage resulted in significantly detrimental effects on *Bacteroidetes*, *Firmicutes* and *Bifidobacterium* spp. in the luminal and mucosal PC of both donors tested. *Lactobacillus* spp. levels were also strongly affected in the luminal PC of donor B, while significantly enhanced lactobacilli levels were observed in the mucosal environment as well as in the proximal lumen and mucus of donor A. In the DC, significantly reduced levels of *A. muciniphila* were observed for both donors tested. In contrast, *Bacteroidetes* levels were boosted in the DC during antibiotic administration in luminal and mucosal environment, with similar effects observed for the *Firmicutes* phylum (except in the lumen of donor B). Overall, upon cessation of antibiotic dosage, microbial activity and community composition recovered towards the end of the 28 days of experimental period.

Growth of *Lacticaseibacillus rhamnosus* GG and *Saccharomyces boulardii*

In order to monitor survivability of the administered test strains selective enumeration was performed in all reactors, thereby focussing on the metabolically active cell fraction of the probiotic strains (Figure 2). The combination of *L. rhamnosus* GG with *S. boulardii* resulted in a superior survival of *L. rhamnosus* GG during the antibiotic period in both colon regions and for both donors tested. Upon cessation of probiotic supplementation, *L. rhamnosus* GG washed out from the system, dropping below detection limit for donor A. In contrast, *S. boulardii* was not affected by antibiotic dosage and nor by *L. rhamnosus* GG presence/absence for both donors tested. Upon cessation of product administration, *S. boulardii* washed out from the system, dropping below detection limit in both colon regions and for both donors tested. For donor A, a faster wash-out was observed in the presence of *L. rhamnosus* GG.

Table 1. Colon-region specific colonisation of microbial community.[1]

Phylum	Family	Donor A				Donor B			
		L		M		L		M	
		PC	DC	PC	DC	PC	DC	PC	DC
Actinobacteria	*Bifidobacteriaceae*	35.38%	*19.93%*	36.39%	*16.44%*	2.80%	2.15%	**11.12%**	3.50%
	Coriobacteriaceae	0.14%	0.38%	0.25%	1.09%	0.06%	*0.12%*	0.22%	4.10%
	Eggerthellaceae	0.00%	0.02%	0.00%	***0.17%***	0.00%	0.04%	0.00%	**0.31%**
	Microbacteriaceae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%
Bacteroidetes	*Bacteroidaceae*	17.41%	*12.66%*	**6.45%**	12.02%	16.81%	*22.96%*	**8.68%**	*23.34%*
	Barnesiellaceae	0.00%	0.00%	0.00%	0.00%	0.00%	*0.06%*	0.00%	*0.11%*
	Marinifilaceae	0.00%	0.01%	0.00%	***0.33%***	0.00%	*0.07%*	0.00%	***0.48%***
	Muribaculaceae	0.00%	0.08%	0.00%	***2.25%***	0.00%	0.00%	0.00%	0.00%
	Prevotellaceae	0.00%	0.11%	0.00%	*0.12%*	0.00%	0.00%	0.00%	0.00%
	Rikenellaceae	0.03%	*0.22%*	0.04%	***1.37%***	0.00%	*0.71%*	0.00%	***1.83%***
	Tannerellaceae	2.04%	2.23%	2.28%	2.09%	0.95%	1.40%	**3.38%**	**1.03%**
Firmicutes	*Acidaminococcaceae*	2.94%	*1.26%*	**0.47%**	**0.44%**	0.00%	0.02%	0.00%	0.01%
	Clostridiaceae_1	0.00%	*0.04%*	0.01%	***4.10%***	0.00%	0.00%	0.00%	4.14%
	Enterococcaceae	0.00%	0.00%	0.03%	0.01%	0.16%	0.05%	0.06%	0.03%
	Erysipelotrichaceae	0.00%	*0.06%*	0.00%	0.32%	0.00%	0.19%	0.00%	***0.46%***
	Eubacteriaceae	0.00%	0.00%	0.00%	0.00%	0.00%	*0.28%*	0.00%	*1.13%*
	Family_XIII	0.00%	*0.11%*	0.00%	***0.61%***	0.00%	*0.09%*	0.00%	*0.15%*
	Lachnospiraceae	35.59%	*31.70%*	35.12%	**42.51%**	11.47%	*19.97%*	**24.72%**	25.98%
	Lactobacillaceae	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.01%
	Peptostreptococcaceae	0.00%	0.00%	0.00%	0.09%	0.00%	0.00%	0.00%	0.00%
	Ruminococcaceae	0.09%	*6.11%*	**0.80%**	*6.52%*	0.03%	*2.51%*	0.30%	***6.17%***
	Veillonellaceae	0.71%	0.41%	**12.61%**	*1.23%*	67.51%	*46.03%*	**51.10%**	**22.26%**
Lentisphaerae	*Victivallaceae*	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.00%	***0.11%***
Proteobacteria	*Burkholderiaceae*	1.46%	0.76%	0.75%	***1.95%***	0.19%	0.66%	0.25%	***1.49%***
	Desulfovibrionaceae	1.97%	1.88%	**3.08%**	2.51%	0.00%	1.65%	0.01%	1.31%
	Enterobacteriaceae	0.67%	0.24%	0.37%	1.50%	0.03%	0.06%	**0.07%**	0.10%
	Pseudomonadaceae	1.00%	1.06%	0.10%	**0.12%**	0.00%	*0.71%*	0.01%	0.23%
	uncultured	0.54%	0.15%	1.21%	**0.06%**	0.00%	0.00%	0.00%	0.00%
	Xanthomonadaceae	0.00%	0.22%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%
Synergistetes	*Synergistaceae*	0.00%	0.02%	0.00%	***0.16%***	0.00%	0.03%	0.00%	***1.25%***
Verrucomicrobia	*Akkermansiaceae*	0.00%	*20.28%*	0.00%	**1.42%**	0.00%	*0.13%*	0.00%	0.14%

[1] Average abundance (%) at microbial family level in the proximal (PC) and distal colon (DC) and the luminal (L) and mucosal environment (M) of the SHIME at the end of the control period for two different human donors (n=4, corresponding to the 4 units within each SHIME) as obtained by 16S-targeted Illumina sequencing. Statistically significant differences between the PC and DC within each environment are underlined, while statistically significant differences between the luminal and mucosal environment within each colonic region are indicated in italics ($P<0.05$). The intensity of green shading indicates the absolute abundance, normalized for each of the different families per donor.

Effects of test products on the microbial metabolic activity

Recovery of microbial metabolic activity following antibiotic dosage was investigated by normalizing SCFA and lactate levels during the final week of the wash-out period to the respective control periods (Table 3). These normalised SCFA profiles resulted in treatment-specific observations. For donor A, a significantly higher production of acetate, propionate and butyrate was observed compared to the antibiotic control incubation (CTRL) upon co-dosing *L. rhamnosus* GG and *S. boulardii* in both colon regions (except for acetate in the PC and butyrate in the DC were no significant effect, but a clear trend was observed). Furthermore, the combination of *L. rhamnosus* GG and *S. boulardii* resulted in synergetic effects as shown

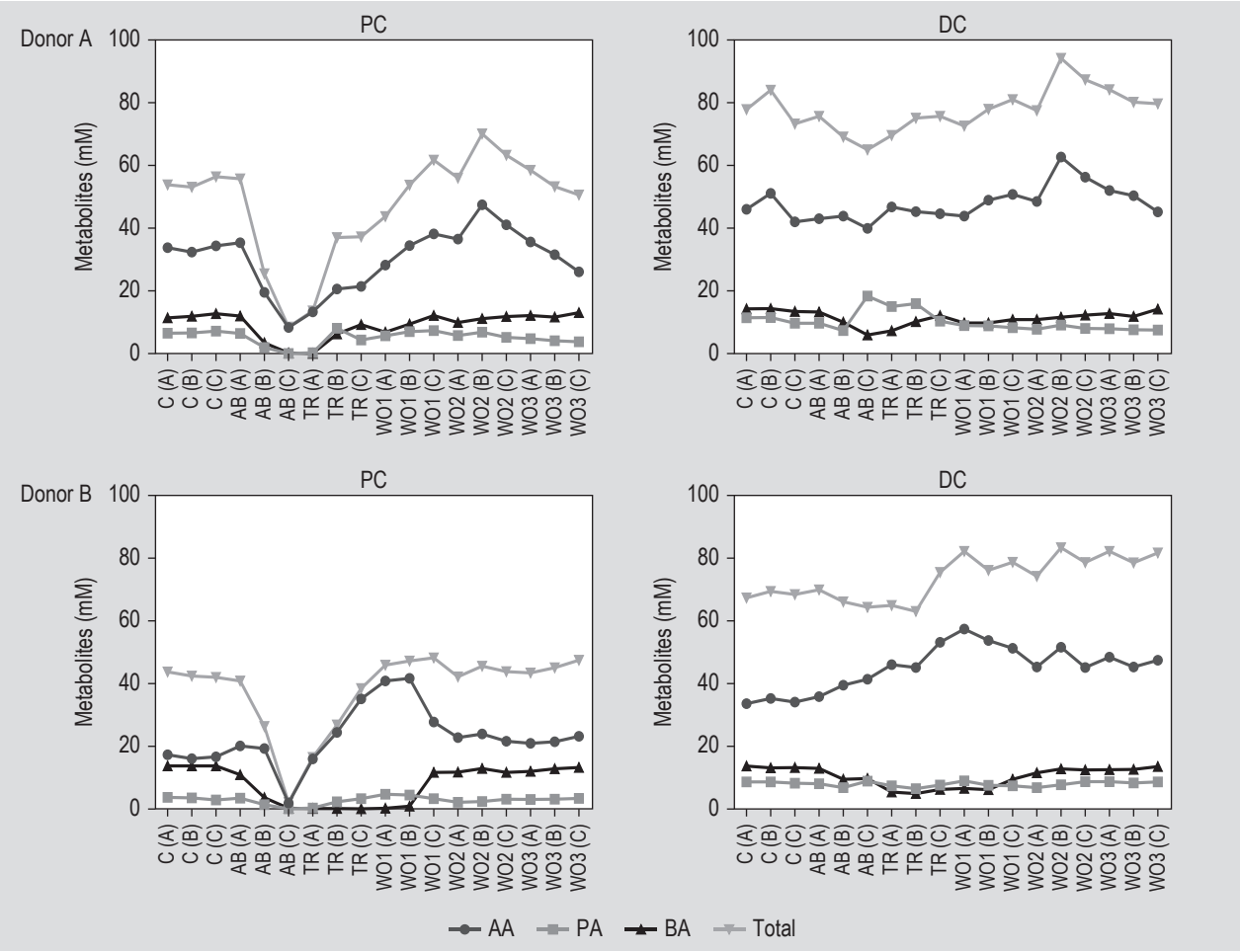

Figure 1. Pervasive effect of antibiotic supplementation on metabolite production. Absolute concentrations (mM) of acetate (AA), propionate (PA), butyrate (BA) and total SCFA in the proximal (PC) and distal colon (DC) reactors for the control unit (CTRL; only receiving antibiotic and thus not supplemented with probiotics during the antibiotic and treatment period) of the SHIME for two different human donors (n=1). Samples were taken one control (C), one antibiotic (AB), one treatment (TR) and three wash-out (WO1, WO2 and WO3) weeks. During each week, three samples (A, B and C) were collected.

by significantly enhanced levels of acetate in the PC, propionate in the DC and butyrate in both colon regions when comparing the respective individual probiotic supplementation with the combined *L. rhamnosus* GG and *S. boulardii* condition. For donor B, all test conditions resulted in acetate levels that were higher compared to the control period, however no differences were observed compared to the antibiotic control incubation (CTRL). Similarly, no significant differences were observed between the different test conditions in terms of propionate production. In contrast, administration of *S. boulardii* resulted in significantly increased butyrate levels compared to the antibiotic control incubation (CTRL) in both colon regions. Finally, dosage of *L. rhamnosus* GG and its combination with *S. boulardii* significantly reduced lactate production compared to the antibiotic control (CTRL) in the DC of donor B. For donor A, no significant differences were observed in terms of lactate production.

Effects of test products on microbial community composition

To assess community composition, a quantitative assessment via qPCR was performed for targeted microbial groups (Figure 3 and Supplementary Figure S2). *L. rhamnosus* GG supplementation (with or without *S. boulardii*) strongly increased luminal and mucosal *Lactobacillus* spp. levels in both colon regions for donor A and B, which was probably a direct consequence of the addition of the *L. rhamnosus* GG strain for these test conditions, thereby indicating that high levels of the probiotic test strain could be maintained in a complex microbial environment. For donor A, levels of *Lactobacillus* spp. also increased upon amoxicillin:clavulanic acid dosage during the antibiotic control incubation (CTRL), however the observed increase was much smaller compared to the strong increase upon *L. rhamnosus* GG supplementation. During the wash-out period, *Lactobacillus* spp. levels decreased (except upon

Table 2. Pervasive effect of antibiotic administration on bacterial groups as assessed by qPCR.[1]

	PC				DC			
	C	AB	TR	WO	C	AB	TR	WO
Donor A								
Akkermansia muciniphila	below LOD	below LOD	below LOD	below LOD	9.33±0.01	**5.90±0.03**	**8.23±0.04**	8.89±0.40
Lactobacillus spp.	4.43±0.29	**7.13±0.04**	**5.32±0.09**	**5.63±0.64**	4.69±0.13	**6.61±0.04**	4.79±0.08	4.75±0.35
Bifidobacterium spp.	9.73±0.04	**8.44±0.00**	9.77±0.02	**10.00±0.10**	9.88±0.03	**9.21±0.01**	9.85±0.07	**9.62±0.11**
Bacteroidetes	9.86±0.04	**6.62±0.02**	9.90±0.05	9.98±0.09	10.07±0.03	**10.55±0.05**	**10.33±0.01**	9.99±0.15
Firmicutes	9.64±0.01	**8.56±0.01**	**9.73±0.04**	**9.84±0.14**	9.98±2.49	**10.07±4.06**	9.97±4.31	9.98±3.29
Donor B		±						
A. muciniphila	below LOD				8.02±0.05	below LOD	**6.72±0.01**	8.14±3.13
Lactobacillus spp.	5.67±0.00	**5.24±0.02**	**6.04±0.02**	5.69±0.14	5.53±0.02	**4.67±0.09**	**5.10±0.13**	**5.17±0.09**
Bifidobacterium spp.	9.10±0.02	**6.61±0.02**	**9.86±0.09**	**9.52±0.29**	9.29±0.03	**8.69±0.01**	**9.73±0.03**	**9.52±0.16**
Bacteroidetes	9.88±0.01	**6.76±0.03**	**10.56±0.05**	9.76±0.21	10.26±0.02	**10.65±0.02**	**10.47±0.05**	10.22±0.11
Firmicutes	9.88±0.01	**9.13±0.01**	**9.98±0.02**	**9.61±0.16**	10.12±0.08	**9.89±0.03**	10.23±0.03	10.04±0.14

[1] Average *Bifidobacterium* spp., *Lactobacillus* spp., *A. muciniphila*, *Bacteroidetes* and *Firmicutes* levels (log 16S rRNA copies/ml) over the control (C; n=1 in technical triplicate), the antibiotic (AB; n=1 in technical triplicate), the treatment (TR; n=1 in technical triplicate) and the wash-out (WO; n=3 in technical triplicate) periods in the luminal proximal (PC) or distal colon (DC) for the control unit (CTRL; only receiving antibiotic) of the SHIME for two different human donors. Data is presented as mean ± standard deviation. Statistically significant differences relative to the control period are indicated in bold (P<0.05). The intensity of green shading indicates the absolute abundance, normalised for each of the different bacterial groups per colonic region. LOD = limit of detection.

S. boulardii administration in donor A), with no notable differences being observed between the different test conditions. Further, antibiotic dosage strongly decreased *Bifidobacterium* spp. levels for both donors tested, especially in the luminal environment. Upon cessation of antibiotic dosage, recovery of *Bifidobacterium* spp. levels was observed in all treated arms, with no notable differences between the different test conditions.

Microbiota profiling at the main site of fermentation (i.e. luminal PC) (Figure 4), using 16S-targeted Illumina sequencing, confirmed the significantly increased abundance of *Lactobacillaceae* upon supplementation of *L. rhamnosus* GG (both in the presence and absence of *S. boulardii*) for both donors tested. Similar results, though less apparent, were observed in the mucosal environment (Supplementary Figure S3). As shown by qPCR analyses, this enrichment proved to be a transient effect, as cessation of product administration resulted in a markedly reduced abundance of *Lactobacillaceae* to levels below the detection limit during the wash-out period. For donor A, antibiotic dosage resulted in reduction of the highly abundant *Bifidobacteriaceae* family, at the expense of *Bacteroidaceae*. Furthermore, *Proteobacteria* were found specifically enriched by antibiotic dosage, as seen by increased abundances of several families belonging to the *Proteobacteria* phylum for all test conditions. Following product administration, the combination of *L. rhamnosus* GG and *S. boulardii* resulted in the strongest increase in *Bifidobacteriaceae* abundance. In the mucosal environment, the increase in *Bifidobacteriaceae* was already observed during the treatment period. Moreover, compared to the antibiotic control (CTRL), supplementation of *S. boulardii* alone or in combination with *L. rhamnsosus* GG resulted in increased levels of *Lachnospiraceae*, mainly at the expense of *Veillonellaceae*. Whereas the microbial community of donor A was strongly enriched in *Bifidobacteriaceae*, the gut microbiota of donor B was characterised by high abundance of *Veillonellaceae*. Antibiotic dosage drastically reduced *Veillonellaceae* levels, as seen by the strong decrease in the antibiotic control incubation (CTRL) during the treatment period in both the luminal and mucosal environment. Remarkably, administration of the different test products alleviated this effect, as *Veillonellaceae* levels remained highly abundant under these test conditions following antibiotic dosage. Following treatment, abundance of several families within the *Proteobacteria* phylum increased for all test conditions, with a specific increase in *Enterobacteriaceae* upon *S. boulardii* supplementation in donor A. Furthermore, some product-specific effects were observed, including increased levels of *Lachnospiraceae* upon supplementation of *L. rhamnosus* GG and co-dosing *L. rhamnosus* GG with *S. boulardii* and enhanced *Bacteroidaceae* levels upon administration of *L. rhamnosus* GG.

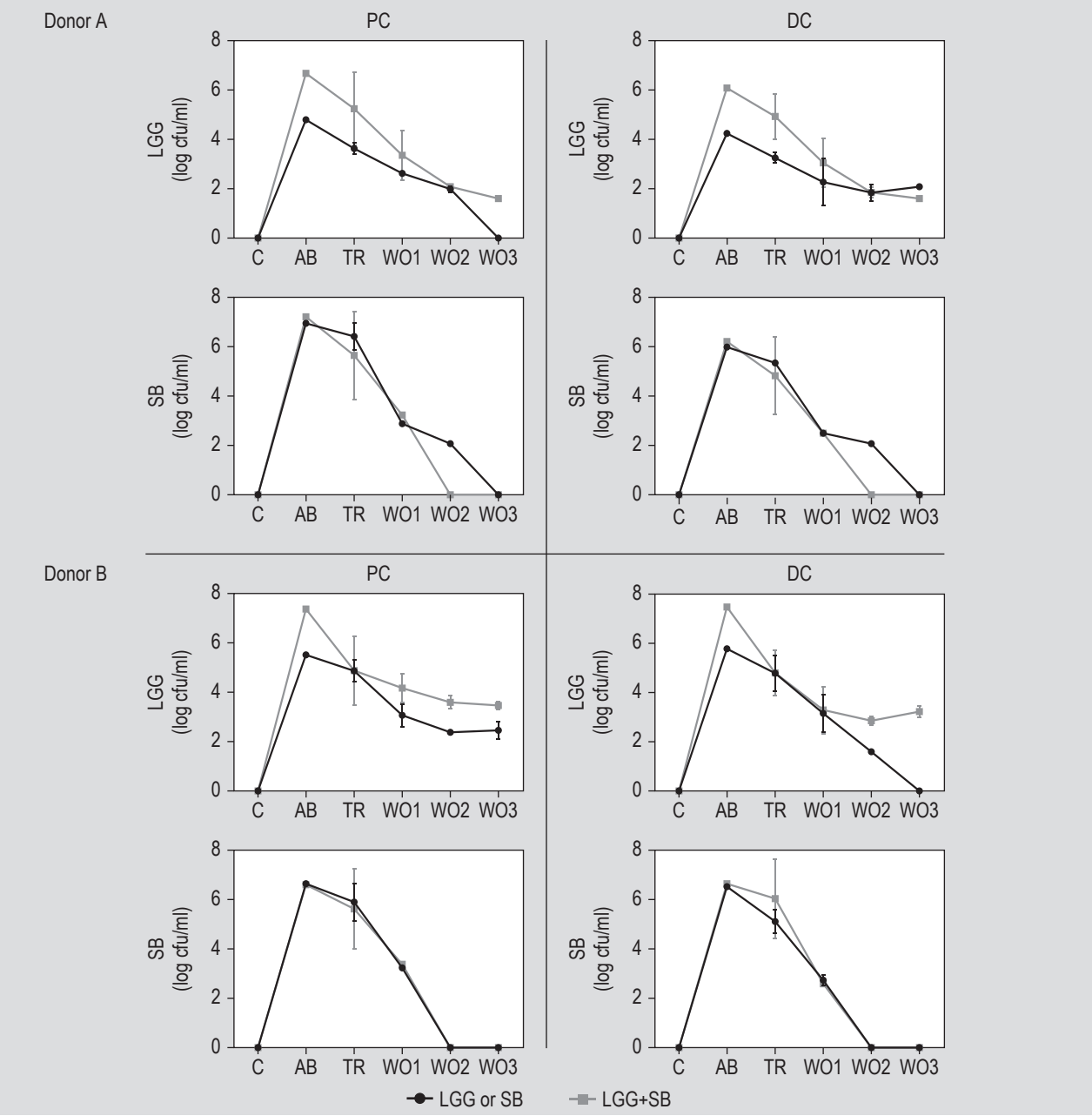

Figure 2. Growth of *Lacticaseibacillus rhamnosus* GG (LGG) and *Saccharomyces boulardii* (SB). Average LGG and SB levels (log cfu/ml) over the control (C), the antibiotic (AB), the treatment (TR) and three wash-out (WO1, WO2 and WO3) weeks in the luminal proximal (PC) or distal colon (DC) upon administration of the individual LGG or SB strain and their combination (LGG+SB) in the SHIME for two different human donors. Data is presented as mean ± standard deviation (n=3).

4. Discussion

During the current study, the impact of repeated doses of *L. rhamnosus* GG, *S. boulardii* and a combination of both strains on recovery of gut microbiota functionality and composition upon co-administration with the antibiotic amoxicillin:clavulanic acid was investigated *in vitro*. For this purpose, the validated M-SHIME model was used, which allows to create a stable microbial community that is representative for the different colonic regions (Liu *et al.*, 2018; Possemiers *et al.*, 2004). The present study confirmed that the M-SHIME model resulted in the creation of a colon region-specific luminal and mucosal microbial community, representative for each of the donors under investigation. Overall, the PC was mainly enriched in bacterial families involved in primary substrate degradation, including *Bifidobacteriaceae* and *Veillonellaceae*, while the environmental conditions in the DC selected for microbial groups with specific metabolic functionalities, such as mucin degradation by *Akkermansiaceae* (Van Herreweghen

Table 3. Recovery of metabolite production.[1]

	PC				DC			
	CTRL	LGG	SB	LGG+SB	C	LGG	SB	LGG+SB
Donor A								
Acetate (mM)	-2.46±4.79 ^{a,b}	-1.92±1.94 ^{a}	1.03±1.39 ^{a}	6.58±1.71 ^{b}	2.81±3.58 ^{a}	6.28±3.75 ^{a,b}	8.87±3.08 ^{a,b}	11.40±2.43 ^{b}
Propionate (mM)	-2.56±0.52 ^{a}	-2.50±0.36 ^{a,b}	-1.39±0.92 ^{a}	0.10±0.83 ^{b}	-3.18±0.24 ^{a}	-3.58±0.38 ^{a}	-3.19±1.00 ^{a}	0.89±0.80 ^{b}
Butyrate (mM)	0.25±0.72 ^{a}	1.50±0.61 ^{b}	2.33±0.18 ^{b}	4.75±0.26	-1.09±1.20 ^{a,b}	-2.49±0.82 ^{a}	-0.75±0.64 ^{a}	2.97±0.53 ^{b}
Lactate (mM)	-0.46±0.10	-0.52±0.06	-0.50±0.02	-0.53±0.02	-0.16±0.06 ^{a}	-0.03±0.07 ^{b}	0.28±0.63 ^{a,b}	1.04±0.83 ^{a,b}
Total SCFA (mM)	-0.33±4.06 ^{a,b}	0.83±2.00 ^{a}	3.37±1.46 ^{a}	12.42±2.36 ^{b}	2.98±2.49 ^{a}	6.90±4.06 ^{a,b}	11.11±4.31 ^{a,b}	16.85±3.29 ^{b}
Donor B								
Acetate (mM)	5.21±1.20	21.54±10.39	8.85±1.90	9.89±3.66	12.84±1.62	15.59±4.26	9.44±5.07	13.19±3.13
Propionate (mM)	-0.21±0.17	2.80±1.64	0.51±0.38	0.57±0.28	0.07±0.22	0.89±0.63	0.45±1.03	-0.03±0.41
Butyrate (mM)	-1.05±0.65 ^{a}	-0.94±0.56 ^{a}	0.64±0.81 ^{b}	-0.09±0.17 ^{a,b}	-0.47±0.53 ^{a}	-1.60±0.49 ^{a}	0.73±0.26 ^{b}	-0.92±0.31 ^{a}
Lactate (mM)	0.29±0.36 ^{a,b}	-0.09±0.16 ^{a}	0.19±0.19 ^{b}	0.39±0.27 ^{b}	-0.02±0.06 ^{a}	-0.26±0.03 ^{b}	0.01±0.18 ^{a,b}	-0.16±0.09 ^{b}
Total SCFA (mM)	2.62±2.03	20.39±11.85	8.85±1.51	8.72±3.49	12.48±2.02	13.33±4.91	9.56±6.07	12.20±2.94

[1] Average normalised concentration of acetate, propionate, butyrate, lactate and total short chain fatty acid (SCFA, mM) associated to the administration of *Lacticaseibacillus rhamnosus* GG (LGG), *Saccharomyces boulardii* (SB) and their combination (LGG+SB) compared to a control (CTRL) in the proximal (PC) and distal colon (DC) during the final wash-out week (WO3) in the SHIME for two different human donors. Data is presented as mean ± standard deviation (n=3). Statistically significant differences between the different conditions in each of the colonic environments are indicated with different letters (P<0.05). The intensity of green shading indicates the absolute abundance, normalised for each of the different metabolites per colonic region.

et al., 2017). This colon-region specificity confirms previous findings by Van den Abbeele *et al.* (2010). Further, the mucosal biofilm has been shown to be specifically colonised by butyrate-producing members of the *Firmicutes* phylum (Van den Abbeele *et al.*, 2013) as well as species from the *Synergistetes* phylum (Liu *et al.*, 2018), as demonstrated during the current study. Overall, the establishment of a representative microbiome in the M-SHIME model provided an excellent tool for performing mechanistic research to unravel effects on the human gut microbiome.

Antibiotic administration strongly affected the *in vitro* microbial community, resulting in diminished levels of SCFA and detrimental effects in terms of community composition. Overall effects were strongest in the PC, probably due to dilution of antibiotic treatment or microbial degradation of amoxicillin:clavulanic acid when moving to the DC. Butyrate production was most strongly affected, showing significant reductions in both colon regions upon amoxicillin:clavulanic acid dosage, confirming findings of *in vitro* work performed by Ladirat *et al.* (2014b). Moreover, the abundance of *Lachnospiraceae* and *Ruminococcaceae*, two families containing several butyrate-producing species, decreased significantly following amoxicillin treatment *in vivo* (Ladirat *et al.*, 2014a; Tsitko *et al.*, 2019), which was for the former bacterial family also observed during the current study in the luminal environment of both donors tested. In concordance with previous *in vivo* studies (Ladirat *et al.*, 2014a; Soldi *et al.*, 2019), *Bifidobacterium* spp. levels were also strongly affected by the amoxicillin:clavulanic acid administration. Propionate production was stimulated in the DC coinciding with enhanced *Bacteroidetes* proportions towards the end of the antibiotic period. While several studies have reported a similar stimulation of several members of the *Bacteroidetes* phylum following amoxicillin administration (Barc *et al.*, 2004; Panda *et al.*, 2014), others showed reduced Bacteroidetes levels as observed in the PC in the current study (Ladirat *et al.*, 2014b; Tsitko *et al.*, 2019). It can be hypothesised that the suppression of *Bifidobacterium* spp. and butyrate-producing microorganisms by antibiotic dosage created a competitive advantage for other bacterial species, including members of the *Bacteroidetes* phylum. This might induce enrichment of the Gram-negative bacterial flora (such as several groups within the *Bacteroidetes* phylum), which has been linked with several side effects such as the induction of diarrhoea (Ramamurthy *et al.*, 2013). Overall, it can be concluded that amoxicillin:clavulanic acid dosage exerted a detrimental effect on the *in vitro* microbial community, in accordance with *in vivo* data, which might be related with the occurrence of AAD.

In order to link the potential effect of *L. rhamnosus* GG and *S. boulardii* on the recovery of the gut microbiome during and following antibiotic supplementation, the presence of viable *L. rhamnosus* GG and *S. boulardii*

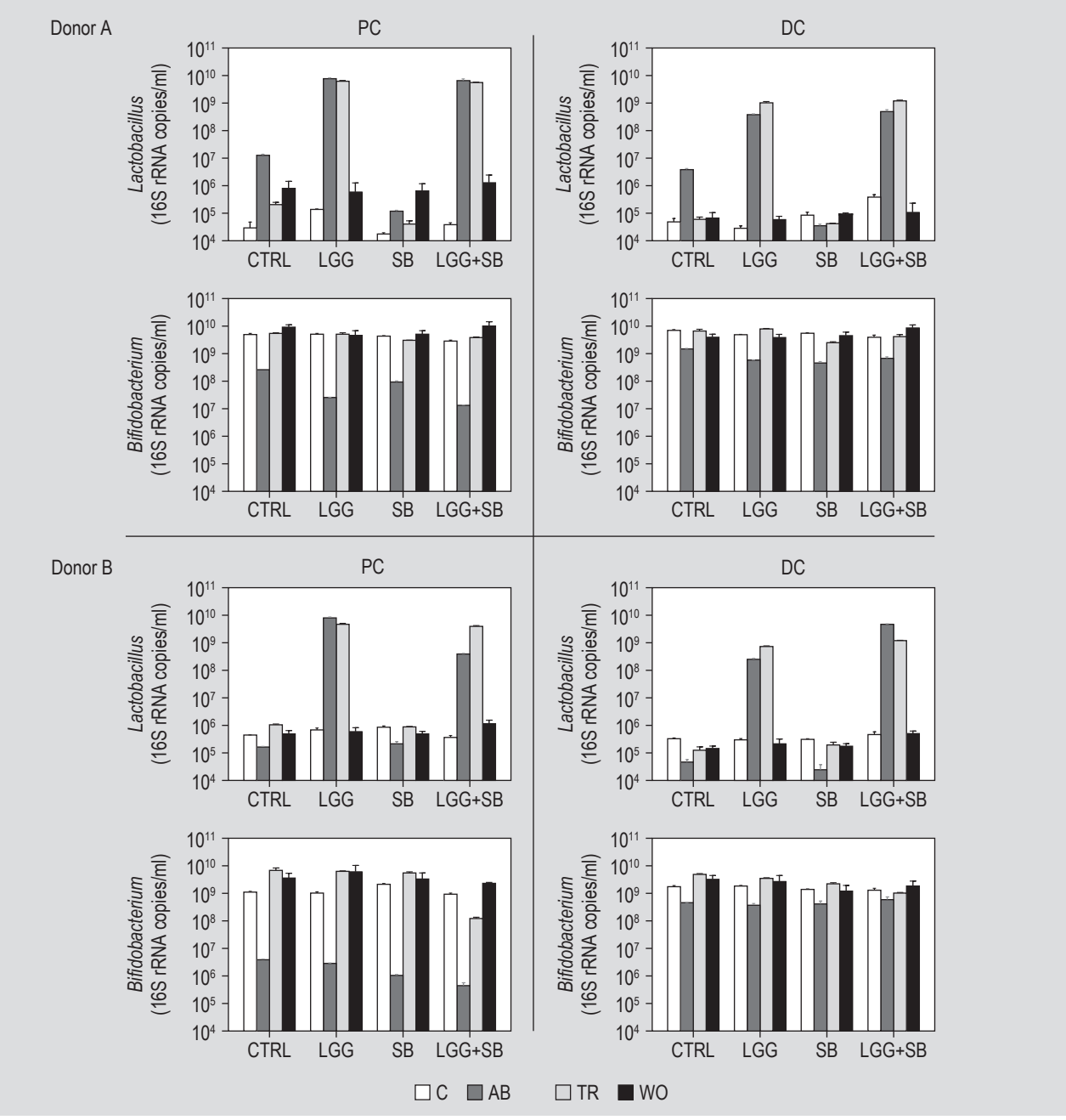

Figure 3. Luminal microbial community composition as assessed via qPCR. Average *Bifidobacterium* spp. and *Lactobacillus* spp. levels (log 16S rRNA copies/mL) associated to the administration of *Lacticaseibacillus rhamnosus* GG (LGG), *Saccharomyces boulardii* (SB) and their combination (LGG+SB) compared to a control (CTRL) in the luminal proximal (PC) or distal colon (DC) during the control (C; n=1 in technical triplicate), the antibiotic (AB; n=1 in technical triplicate), the treatment (TR; n=1 in technical triplicate) and the wash-out (WO; n=3 in technical triplicate) periods in the SHIME for two different human donors. Data is presented as mean ± standard deviation.

cells was assessed throughout the experimental period. Selective enumeration pointed out that viable *L. rhamnosus* GG cells were specifically detected in the SHIME units where *L. rhamnosus* GG was administered. The survival of *L. rhamnosus* GG during antibiotic dosage was enhanced upon co-supplementation with *S. boulardii* versus the unit where *L. rhamnosus* GG was administered alone, and this in both colon regions and for both donors tested. Recently, it was shown by our research group that no antagonistic effects between *L. rhamnosus* GG and *S. boulardii* were observed when cultivated together (Moens *et al.*, 2019). The current study further complements these observations, showing protective effects of *S. boulardii* on survival of *L. rhamnosus* GG during antibiotic dosage. Further, high

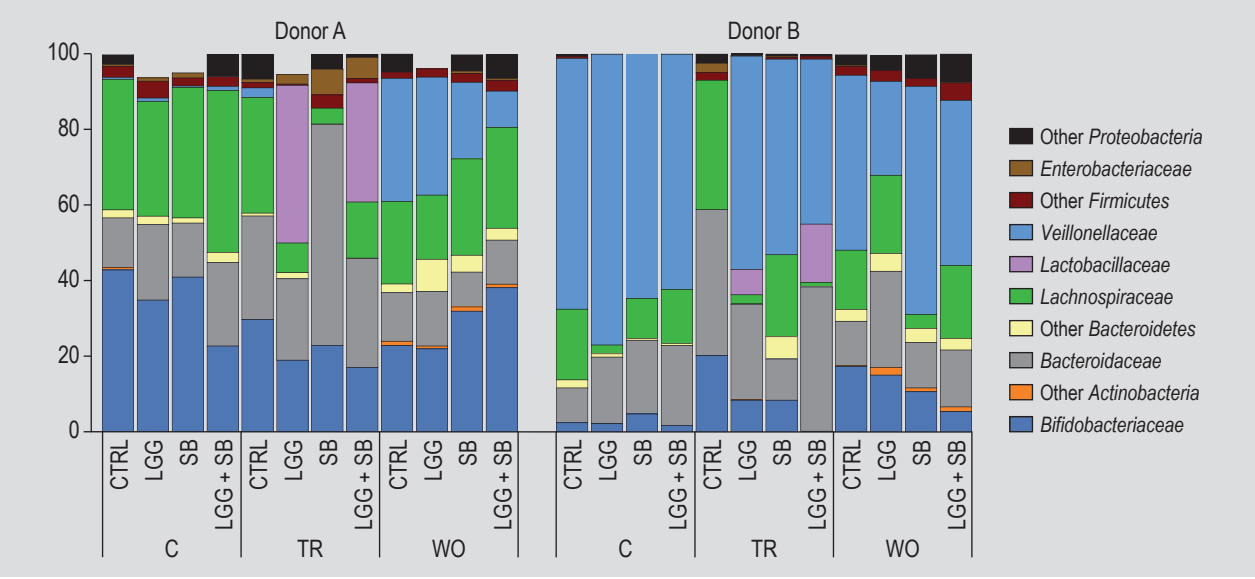

Figure 4. Luminal microbial community composition as assessed via 16S-targeted Illumina sequencing. Abundance (%) at microbial family level associated to the administration of *Lacticaseibacillus rhamnosus* GG (LGG), *Saccharomyces boulardii* (SB) and their combination (LGG+SB) compared to a control (CTRL) in the luminal proximal colon (PC) at the end of the control period (C), treatment period (TR) and wash-out period (WO) in the SHIME for two different human donors (n=1).

levels of viable *S. boulardii* cells were detected in the SHIME units where *S. boulardii* was administered and these levels were not affected by the antibiotic dosage, confirming literature findings (Neut *et al.*, 2017), regardless of the presence/absence of *L. rhamnosus* GG. Upon cessation of the product administration, both test strains washed out from the system. It has been shown that persistence of individual strains varies in a strain-specific manner *in vivo* (Tremblay *et al.*, 2020). Indeed, while *S. boulardii* is only to persist in the colonic environment for hours upon cessation of dosing, *L. rhamnosus* GG can persevere for several weeks in the colonic environment (McFarland and Elmer, 2005). Overall, these findings demonstrated that the strains were not only dosed but also capable to thrive in both colon regions of both donors tested, thus potentially resulting in beneficial effects on antibiotic-induced dysbiosis.

The impact of the probiotic test strains on the recovery of microbial functionality and composition showed donor-dependent differences. 16S-targeted Illumina sequencing revealed that supplementation of *L. rhamnosus* GG and/or *S. boulardii* resulted in a protective effect on the most abundant bacterial groups within the bacterial community of each donor upon antibiotic dosage. Donor A was mainly characterised by high proportions of *Bifidobacteriaceae* and *Lachnospiraceae*, with both bacterial groups being highly affected by antibiotic dosage. Upon cessation of antibiotic administration, *Lachnospiraceae* recovered in all test conditions, with higher levels observed upon probiotic supplementation, especially for addition of *S. boulardii* and its combination with *L. rhamnosus* GG. Supplementation of *S. boulardii* has been reported to lead to a faster recovery of the gut microbiome following a state of dysbiosis, including positive effects on *Lachnospiraceae* levels (Moré and Swidsinski, 2015). Furthermore, co-dosing *L. rhamnosus* GG and *S. boulardii* resulted in superior SCFA recovery compared to the antibiotic control, accompanied by a stronger increase in abundance of *Bifidobacteriaceae*. This confirmed previous results from our research group, which demonstrated that combination of *L. rhamnosus* GG and *S. boulardii* improved microbiota functionality under impaired conditions (Moens *et al.*, 2019). Donor B instead was characterised by a higher abundance of *Veillonellaceae* in its healthy state, levels of which were drastically reduced following antibiotic dosage in the control arm, while supplementation of the different probiotic test strains preserved *Veillonellaceae* levels, indicating a potential protective effect of *L. rhamnosus* GG and *S. boulardii* on *Veillonellaceae* levels during antibiotic dosage for this particular donor. Collectively, these results indicate that effects of antibiotic co-treatment with *L. rhamnosus* GG and *S. boulardii* on gut microbiota functionality and composition may differ substantially depending on the baseline composition of the gut microbiota of each subject, although reinforcing the hypothesis that combined intake of *L. rhamnosus* GG and *S. boulardii* might stimulate microbiome recovery following antibiotic dosage.

Some potential limitations of the study are acknowledged. While inter-individual variations were evaluated, only two donors were selected for the current study. However, it is important to note that the human microbial community composition is generally characterised by large inter-individual differences (Eckburg *et al.*, 2005) and this

should therefore also be taken into account upon using *in vitro* gut models. It has been reported that the gut microbial community of healthy individuals naturally recovers following antibiotic administration, reaching a near-baseline composition within 45 days following a short antibiotic intervention period (Palleja *et al.*, 2018). In the current study, a parallel antibiotic control was included to map natural microbiome recovery following a 5-day amoxicillin:clavulanic acid dosage *in vitro*. Near-full recovery of microbial metabolic activity and community composition was observed during the post-intervention period of 28 days. This fast recovery of the microbial community during the present *in vitro* study might therefore have obscured potential effects of *L. rhamnosus*, *S. boulardii* and their combination on microbiome recovery during the post-intervention period. Furthermore, while the gut microbial community generally recovers to its healthy state in 45 days due to its resilience, long-term alterations may persist in some subjects explaining susceptibility to AAD or other dysbiosis-related diseases (De La Cochetière *et al.*, 2005; Sommer and Bäckhed, 2013). Furthermore, host-microbiome interactions were not investigated during the current study. While the current study has shown that not only the luminal microbial community was affected by the administered test products, but that also the mucus-associated microbiota was impacted, these results point to potential interesting interactions with the intestinal epithelial cells. Combining samples obtained from the SHIME model with a co-culture human cell model (Daguet *et al.*, 2016), could have provided insight on the immune-modulatory properties of *L. rhamnosus*, *S. boulardii* and their combination following antibiotic dosage.

In conclusion, administration the antibiotic amoxicillin: clavulanic acid exerted a detrimental effect on the human gut microbiome in the simulated gastrointestinal tract. While recovery of the microbial community was observed at the end of the 28d experimental period, supplementation of *L. rhamnosus* GG and *S. boulardii* positively impacted colonic balance, though in a donor-dependent fashion. Evidence was provided that co-dosing of *L. rhamnosus* GG and *S. boulardii* might additionally stimulate recovery of microbial functionality and composition following amoxicillin:clavulanic acid dosage, which could provide an interesting approach for antibiotic-related gastrointestinal symptoms in the clinical environment such as in the protection against AAD. However, future research is warranted to confirm these findings.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2020.0180.

Figure S1. Design of SHIME experiment.

Figure S2. Mucosal microbial community composition as assessed via qPCR.

Figure S3. Mucosal microbial community composition as assessed via 16S-targeted Illumina sequencing.

Table S1. Pervasive effect of antibiotic administration on mucosal bacterial groups as assessed by qPCR.

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Conflict of interest

Mireia Morera is an employee of IPSEN Consumer HealthCare SAS.

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