

# ***Lactobacillus rhamnosus* GG and *Saccharomyces cerevisiae boulardii* exert synergistic antipathogenic activity *in vitro* against enterotoxigenic *Escherichia coli***

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## Abstract

Short-term colonic *in vitro* batch incubations were performed to elucidate the possible synergistic effects of *Lactobacillus rhamnosus* GG (CNCM-I-4798) and *Saccharomyces cerevisiae boulardii* (CNCM-I-1079) (associated in Smebiocta/Smectaflora Protect®) on the colonic microbial fermentation process, as well as their antipathogenic activity against enterotoxigenic *Escherichia coli* (LMG2092) (ETEC). These incubations adequately simulate the native microbiota and environmental conditions of the proximal colon of both adult and toddler donors, including the colonic mucosal layer. Results indicated that both strains were capable of growing together without showing antagonistic effects. Co-cultivation of both strains resulted in increased butyrate (stimulated by *L. rhamnosus* GG), propionate (stimulated by *S. boulardii*), and ethanol (produced by *S. boulardii*) production compared to the control incubations, revealing the additive effect of both strains. After inoculation of ETEC under simulated dysbiotic conditions, a 40 and 46% reduction in the concentration of ETEC was observed upon addition of both strains during the experiments with the adult and toddler donor, respectively. Furthermore, ETEC toxin levels decreased upon *S. boulardii* inoculation, probably due to proteolytic activity of this strain, with a synergistic effect being observed upon co-cultivation of *L. rhamnosus* GG and *S. boulardii* resulting in a reduction of 57 and 46% for the adult and toddler donor, respectively. Altogether, the results suggest that both probiotics together may help microbiota functionality, in both adults and toddlers and under healthy or impaired conditions, which could be of great interest when the colonic microbiota is dysbiotic and therefore sensitive to pathogenic invasion such as during antibiotic treatment.

**Keywords:** LGG, yeast, ETEC, gut microbiota, dysbiosis

## 1. Introduction

Probiotics are recognised for their role in improving human health, most likely by modifying the gut microbiota composition in a transient manner. Indeed, probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (Hill *et al.*, 2014). Among the most documented probiotic strains, *Lactobacillus rhamnosus* GG (Doron *et al.*, 2005) has been associated with several health-promoting properties including prevention of antibiotic-induced diarrhoea (Vanderhoof *et al.*, 1999), recovery from disease-related types of diarrhoea (Guandalini *et al.*, 2000; Szajewska *et al.*, 2011), prevention of atopic disease (Kalliomaki *et al.*, 2001),

improvement of immune function (Pena and Versalovic, 2003; Pessi *et al.*, 2000) and antipathogenic activity (De Keersmaecker *et al.*, 2006) due to the production of antimicrobial compounds. For example, the antimicrobial activity of *L. rhamnosus* GG against the intestinal pathogen *Salmonella typhimurium* has been linked to lactic acid secretion into the culture medium (De Keersmaecker *et al.*, 2006). Another group investigating the antipathogenic effect of *L. rhamnosus* GG against the respiratory tract pathogen *Moraxella catarrhalis* obtained similar results (Van den Broek *et al.*, 2018). Another extensively used health-promoting microorganism is the non-pathogenic yeast *Saccharomyces cerevisiae boulardii*. Yeasts are resistant to antibiotics and therefore are frequently used as preventive

and therapeutic agents for antibiotic-associated diarrhoea (Czerucka *et al.*, 2007). Furthermore, *S. boulardii* has shown excellent survival through the upper gastro-intestinal tract, which is an important characteristic of probiotic products intended for oral consumption (Tompkins *et al.*, 2011). Several mechanisms of action have been described for the health-promoting activity of *S. boulardii*, including regulation of intestinal microbial homeostasis, regulation of immune response, improvement of gut barrier function, antitoxin effects and antimicrobial activity (Kelesidis and Pothoulakis, 2012). *S. boulardii* has been shown to exert antipathogenic activity against several disease-causing *Escherichia coli* strains, such as enterohemorrhagic *E. coli* (EHEC) (Dahan *et al.*, 2003; Dalmasso *et al.*, 2006), enteropathogenic *E. coli* (EPEC) (Czerucka *et al.*, 2000) and enterotoxigenic *E. coli* (ETEC) (Badia *et al.*, 2012).

ETEC infection is recognised as one of the most common causes of traveller's diarrhoea and of childhood diarrhoea in developing countries (Anonymous, 2006). Furthermore, foodborne outbreaks of ETEC infection have emerged in developed countries, such as the USA (Dalton *et al.*, 1999), Norway (MacDonald *et al.*, 2015), Denmark (Pakalinskiene *et al.*, 2009) and South Korea (Shin *et al.*, 2016). One of the major virulence factors of ETEC infection includes adherence of the pathogenic strain to the epithelium of the small intestine, which is mediated by several colonisation factors including numerous fimbriate surface antigens (Gaastra and Svennerholm, 1996). Colonisation of the small intestine is followed by the release of either or both heat-stable and heat-labile enterotoxins, causing diarrhoea (Qadri *et al.*, 2005). The resulting infection generally lasts for several days and ranges from illness with mild diarrhoea to severe cholera-like disease (Anonymous, 2006). Several studies have reported the potential role of probiotics in the prevention and/or treatment of ETEC infections (Roselli *et al.*, 2006; Tsai *et al.*, 2008). *L. rhamnosus* GG was shown to partially impair ETEC adhesion to intestinal epithelial cells *in vitro*, probably through competition for binding sites and/or secretion of a non-proteinaceous antimicrobial metabolite (Roselli *et al.*, 2006). Additionally, *L. rhamnosus* GG exerted anti-inflammatory properties in response to ETEC-induced inflammation by regulation of chemokine and cytokine expression. Furthermore, *S. boulardii* was shown to prevent ETEC infection *in vitro* by preventing ETEC from adhering to intestinal epithelial cells and reducing pathogenic inflammation via secretion of anti-inflammatory factors (Badia *et al.*, 2012). As such, co-administration of *L. rhamnosus* GG together with *S. boulardii* could therefore potentiate the beneficial effects of these probiotics on ETEC infection.

Further, since most studies regarding the interactions of probiotics with ETEC have been performed *in vitro* and focused on the small intestinal environment, only a limited amount of data exists on the interactions of ETEC

with the colonic microbiota and probiotics (Roussel *et al.*, 2017a,b). In humans, the colonic microbiota plays a predominant role on the possible invasion of ETEC and induction of disease (Pop *et al.*, 2016). Indeed, microbiota compositional analysis revealed an association between the presence of 12 Operational Taxonomic Units (OTUs) in the microbiota and the absence of development of diarrheal disease upon ETEC challenge. Similar results were obtained during studies with piglets challenged with ETEC (Bin *et al.*, 2018). Microbiome profiling has revealed that travellers who develop diarrhoea upon ETEC infection have a dysbiotic colon microbiota that is characterised by a high *Firmicutes:Bacteroidetes* ratio (Youmans *et al.*, 2015). As such, modulation of the gut microbiota and its associated fermentation process through the ingestion of probiotics could be used as a strategy to protect against ETEC infection. Indeed, *L. rhamnosus* GG has been shown effective at preventing post-weaning diarrhoea in piglets, which was associated with modulation of the intestinal microbiota (Zhang *et al.*, 2010). Modulation of the gut microbiota in newly weaned pigs through a select mixture of *Bacillus* species has been shown to improve ETEC-induced enteritis symptoms (Zhang *et al.*, 2017). Finally, pre-supplementation of the probiotic strain *Lactobacillus reuteri* HCM2 to mice prevents the dysbiosis of the colonic microbiota caused by ETEC through modulation of the gut microbial community (Wang *et al.*, 2018).

The main objective of this study was to determine how *L. rhamnosus* GG and *S. boulardii*, when administered together, would affect the colonic microbial fermentation process and identify their respective and combined activity against ETEC. We hypothesised that these strains could possibly act synergistically when added together to a healthy and altered colonic microbiota environment *in vitro*.

## 2. Materials and methods

### Chemicals and test products

All chemicals were obtained from Sigma-Aldrich (Overijse, Belgium) unless stated otherwise. Lallemand Health Solutions Inc. (Blagnac, France) provided the test strains *Saccharomyces cerevisiae boulardii* (CNCM-I-1079) and *Lactobacillus rhamnosus* GG (CNCM-I-4798) associated in Smebiocta/Smectaflora Protect<sup>®</sup>. Enterotoxigenic *Escherichia coli* LMG2092 (ETEC) was obtained from BCCM/LMG (Ghent, Belgium).

### Short-term colonic microbiota batch incubations

To account for inter-individual differences, two different donors, an adult and a toddler, were used as a source of colonic background community. Under healthy conditions for both donors, the short-term colonic batch incubations were performed in 120 ml penicillin bottles and initiated

by the addition of 63 ml colonic background medium containing host- and diet-derived nutritional compounds. Four types of colonic incubations were performed. During the control (CTRL) incubations only the colonic background microbiota was added. During the 'LGG' experiments  $10^9$  cfu of *L. rhamnosus* GG were dosed to the incubations together with the colonic background microbiota. During the 'SB' experiments 50 mg of *S. boulardii* powder was added to the colonic incubations containing a metabolically active colonic microbiota. Both *L. rhamnosus* GG, *S. boulardii* and a colonic background microbiota were dosed to the 'LGG + SB' incubations. Background microbiota were obtained from both a healthy adult donor and from a healthy 3-year old toddler donor. Briefly, faecal samples were freshly collected in containers containing an AnaeroGen sachet to maintain anaerobic atmosphere. A faecal inoculum was prepared by making a 1:13 (w/v) mixture of the faecal sample with anaerobic phosphate buffer ( $K_2HPO_4$  8.8 g/l;  $KH_2PO_4$  6.8 g/l; sodium thioglycolate 0.1 g/l; sodium dithionite 0.015 g/l). After homogenisation (10 min, BagMixer<sup>®</sup> 400, Interscience, Louvain-La-Neuve, Belgium) and removal of big particles via centrifugation (2 min,  $500\times g$ ), an inoculum corresponding to 10% (v/v) was added to the different incubations. Furthermore, during all incubations the mucosal layer of the colon was simulated by the addition of mucus beads (Van den Abbeele *et al.*, 2012). Briefly, mucus beads were prepared by covering microcosms (Kaldness K1, AnoxKaldnes AB, Lund, Sweden) with a mucin agar solution and combining them in a polyethylene netting (Zakkencentrale, Rotterdam, the Netherlands) at a ratio of 1 microcosm per 9 ml of colonic medium. All incubations were performed under strict anaerobic conditions. Anaerobiosis was obtained by flushing with  $N_2$ . All experiments were incubated at 37 °C for a period of 48 h under continuous mixing (90 rpm). All experiments were performed in biological triplicate.

Under dysbiotic conditions, colonic incubations were performed in a similar way, with the following modifications: during all incubations, only 0.02% (v/v) of background colonic microbial community was added in order to simulate dysbiosis. Furthermore, 2% (v/v) of an overnight ETEC culture was added to all incubations, resulting in approximately 7.5 log ETEC/ml at the start of the incubations. ETEC was grown overnight in nutrient broth at 37 °C under normal atmospheric conditions and continuous shaking (110 rpm). The overnight culture was subsequently centrifuged at  $1,500\times g$  for 10 min, the supernatant was removed and the pellet was resuspended in sterile phosphate-buffered saline (PBS).

### Microbial growth

Quantification of the viable microbial cells of *L. rhamnosus* GG, *S. boulardii*, and ETEC during the colonic batch incubations was determined by generating cell pellets

at different time points during the incubation (baseline, 6 h, 24 h, and 48 h) which were subjected to propidium monoazide (PMA, Biotium, Hayward, CA, USA) treatment. Briefly, 50 µl of sample was added to 450 µl of sterile anaerobic peptone water. PMA was added to reach a final concentration of 50 µM. Samples were shaken in the dark for 5 min at room temperature. Afterwards the PMA-treated cell suspensions were placed in a LED-Active Blue system (Ingenia Biosystems, Barcelona, Spain) for 15 min followed by centrifugation for 5 min at  $7,690\times g$ . Cell pellets were stored at -20 °C. Subsequently, DNA was extracted from these PMA-treated cells using the DNeasy UltraClean Microbial Kit (QIAGEN, Venlo, the Netherlands) according to manufacturer's instructions, followed by quantification of their concentration through qPCR with a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using primers specific for *L. rhamnosus* GG (Brandt and Alatossava, 2003), *S. cerevisiae* (Zott *et al.*, 2010) and ETEC (Taniuchi *et al.*, 2012). The qPCR protocols of *L. rhamnosus* GG and *S. cerevisiae* were optimised in order to allow quantification of both strains in the presence of a complex background microbiota. The qPCR assays resulted in an efficiency of 77 and 85% for *L. rhamnosus* GG and *S. cerevisiae* respectively, with good reproducibility between biological replicates. The mucin agar of the mucus beads was harvested after 48 h of incubation. DNA extraction was performed on 0.25 g of mucus followed by specific qPCR to study the adhesion of the strains to the mucosal layer.

### ETEC toxin determination

The concentration of heat-labile toxin produced by ETEC was determined after 0, 6, 24, and 48 h of colonic incubation through GM1 ELISA as described previously by Salimian *et al.* (2010) with some minor modifications. Absorbance was measured using the Synergy HT Microplate reader (BioTek, Bad Friedrichshall, Germany). Both the concentration of toxin present in the fermentation liquid as well as the concentration of toxin that was bound to the cell wall were determined by separating the supernatant and cell pellet originating from 1 ml of sample. The cell pellet was resuspended in PBS and sonicated prior to toxin quantification.

### Microbial metabolic activity

Short-chain fatty acids (SCFA), lactate, ammonium and ethanol production were measured at the start of the incubation, and after 48 h. Lactate quantification was performed using a commercially available enzymatic assay kit (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions. Ammonium analysis was performed using a KjelMaster K-375 device (Büchi, Hendrik-Ido-Ambacht, the Netherlands). Ammonium in the sample was liberated as ammonia by the addition of

32% NaOH. The released ammonia was distilled from the sample into a 2% boric acid solution. Finally, the ammonium in the distillate was determined by titration with a 0.02 M HCl solution. Short chain fatty acids (SCFA), including acetate, propionate, butyrate and branched SCFA (sum of isobutyrate, isovalerate and isocaproate), and ethanol were extracted by the addition of 0.1 g of NaCl and an acetonitrile mixture, containing 99.5% acetonitrile and 0.5% formic acid, in a 1:1 ratio. As an internal standard, 2-methyl hexanoic acid was added to all samples. Suspensions were centrifuged for 15 min at 15,080×g after which concentrations of SCFA and ethanol were determined using a GC-2014 gas chromatograph with flame ionisation (FID) (Shimadzu, 's-Hertogenbosch, the Netherlands). Nitrogen was used as a carrier gas at a constant flow rate of 2.19 ml/min and as a make-up gas. The injector and detector temperatures were set at 200 and 240 °C, respectively. The column temperature program was as follows: 0 min 40 °C; 18 min 220 °C and 23 min 220 °C. The injection volume was 1 µl and a split ratio of 40 was applied.

### Agar well diffusion assay

First, second-generation cultures of ETEC, *L. rhamnosus* GG, and *S. boulardii* were prepared in Luria-Bertani (LB) medium, De Man-Rogosa-Sharp (MRS) medium, and yeast extract peptone dextrose (YEPD) medium, respectively, by growing them during each generation for 24 h at 37 °C under normal atmospheric conditions. *L. rhamnosus* GG and *S. boulardii* cells were harvested and four aliquots were prepared: (1) 3 ml of the second-generation culture was centrifuged for 5 min at 1,500×g, the supernatant (pH of 4.4 for *L. rhamnosus* GG and 5.5 for *S. boulardii*) was collected and subsequently filtered (pore size of 0.2 µm) into a sterile recipient; (2) 3 ml of the second-generation culture was centrifuged for 5 min at 1,500×g, the supernatant was collected and pH adjusted to 6.5 using 1M NaOH or 1M HCl; (3) 3 ml of the second-generation culture was sonicated for 2 min; (4) 2 ml of the second-generation culture was centrifuged for 5 min at 5,000×g, and the pellet was resuspended in an equal amount of PBS. ETEC was prepared by centrifuging 2 ml of a second-generation culture for 5 min at 1,500×g. The resulting cell pellet was resuspended in 6 ml of sterile PBS to obtain an ETEC solution at 10<sup>8</sup> cfu/ml.

The agar well diffusion assay was performed by first adding 0.5 ml of the resuspended ETEC culture to big petri dishes followed by the addition of 20 ml of YEPD agar medium. The plates were swung to mix ETEC equally over the agar plate. After drying the plates, were subsequently dried. Afterwards holes were punched in the solidified agar using a sterile Pasteur pipet to create wells. The four test aliquots of *L. rhamnosus* GG and *S. boulardii* were mixed with an equal amount of 2× concentrated molten MRS agar medium and 2× concentrated molten YEPD agar medium,

respectively. The wells in the YEPD agar plates seeded with ETEC, were filled with the molten agar media containing the test solutions. The plates were allowed to dry and were subsequently incubated for 24 h at 37 °C under normal atmospheric conditions.

### Statistics

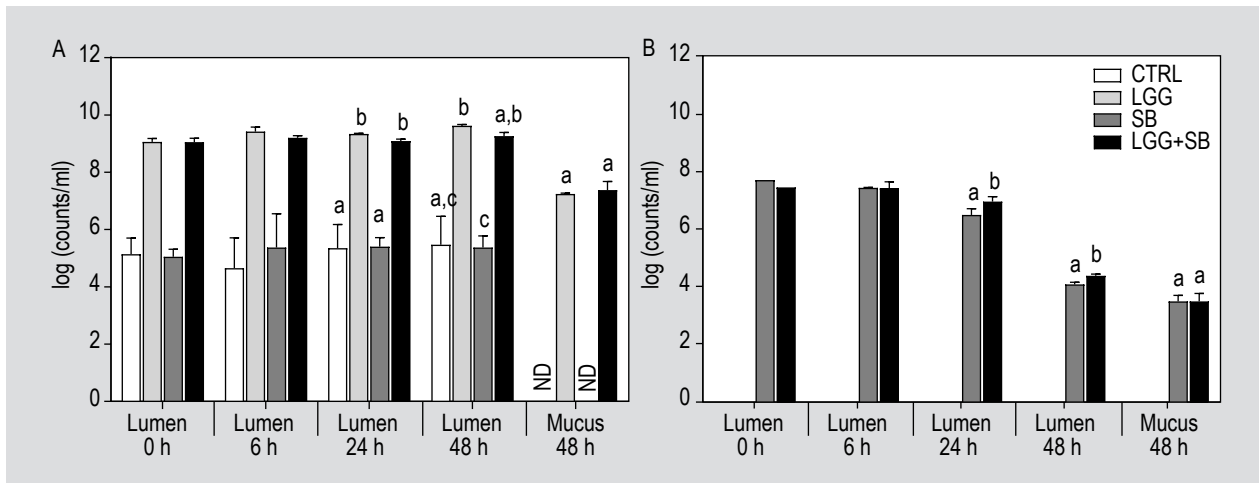
All experiments were performed in biological triplicate. Statistical analysis was performed using the SPSS Statistics software, version 25 (SPSS, Chicago, IL, USA). Normality of data and equality of the variances were determined with a Shapiro-Wilk test and a Levene's test, respectively. For normally distributed data with equal variances a one-way ANOVA with a Bonferroni post-hoc test was used. For normally distributed data with unequal variances, a Welch test with a Games-Howell post-hoc test was conducted. For non-normally distributed data, a Kruskal-Wallis one-way ANOVA test with multiple post-hoc pairwise comparisons were performed. Bonferroni corrections were implemented on the significance levels of the multiple pairwise comparisons. In terms of statistics, the differences for all data discussed and indicated by '*P*<0.05' were significant with a confidence interval of 95%.

## 3. Results

### Growth of *Lactobacillus rhamnosus* GG and *Saccharomyces c. boulardii*

Growth of *L. rhamnosus* GG and *S. boulardii* was measured in the presence of a healthy colonic background microbiota of both an adult (Figure 1) and a toddler (Supplementary Figure S1) donor. Similar effects were observed for both donors. During control incubations, as well as during the incubations to which *S. boulardii* was added, low background levels of *L. rhamnosus* GG were detected throughout the incubation period. Addition of *L. rhamnosus* GG to the incubations (alone or in combination with *S. boulardii*) resulted in initial concentrations of 9.1 log cfu/ml, followed by moderate increases up to a maximum concentration of 9.7 log cfu/ml throughout the incubation period. On the contrary, inoculating *S. boulardii* to the incubations resulted in initial concentrations of 7.7 log cfu/ml when *S. boulardii* was inoculated alone, and 7.4 log cfu/ml when *S. boulardii* was supplemented in combination with *L. rhamnosus* GG, followed by a reduction towards the end of the incubation period. In the adult model, significantly higher concentrations of *S. boulardii* were present after 24 and 48 h of incubation compared to the incubations that were performed with *S. boulardii* alone.

The possible adhesion of *L. rhamnosus* GG and *S. boulardii* to the mucosal layer was studied by the addition of mucin-coated beads to the colon medium. Supplementation of *L. rhamnosus* GG resulted in the presence of 7.2 log cfu/ml



**Figure 1.** Growth of (A) *Lactobacillus rhamnosus* GG (LGG) and (B) *Saccharomyces boulardii* (SB) in microbiota colonic incubations from an adult donor. Mean log count/ml ( $\pm$  standard deviation) of LGG and in the luminal and mucosal environment at several time points during the short-term colonic incubations using the colonic background microbiota of an adult donor ( $n=3$ ). LGG and SB were tested separately as well as together (LGG+SB) compared to a control incubation (CTRL). Statistically significant differences between experimental conditions (i.e. CTRL, LGG, SB and LGG+SB) after 24 and 48 h of incubation are highlighted by assigning different letters ( $P<0.05$ ). ND = not detectable.

for the adult donor and 6.8 log cfu/ml for the toddler donor after 48 h of incubation. Mucosal binding of *S. boulardii* was lower compared to *L. rhamnosus* GG, reaching concentrations of 3.5 log cfu/ml and 3.8 log cfu/ml for the adult and toddler donor, respectively. When *L. rhamnosus* GG and *S. boulardii* were supplemented together, a similar adhesion of the mucosal layer was observed.

#### Microbial activity of *Lactobacillus rhamnosus* GG and *Saccharomyces c. boulardii* under healthy and dysbiotic conditions

During the control incubations with the adult donor, an overall SCFA production of 86.5 mM, mainly attributed to acetate, propionate and butyrate, was observed under healthy conditions (Table 1), which indicated the high metabolic activity of the donor microbiota. When *L. rhamnosus* GG was added to the incubations, acetate levels significantly decreased compared to the control incubations. This coincided with statistically significant higher levels of butyrate during these incubations, i.e. 25.0 mM compared to 17.0 mM for the control incubations. During the incubations with *S. boulardii* significantly more propionate was produced compared to the control experiments. Administration of both *L. rhamnosus* GG and *S. boulardii* to the colonic microbiota resulted in higher concentrations of both butyrate and propionate compared to the control experiments, indicating the additive activity of both strains on the colonic microbiota.

Under dysbiotic conditions (Table 2), SCFA production was significantly lower during the control incubations compared to the healthy conditions ( $P=0.008$  for acetate,

$P=0.009$  for propionate and  $P<0.001$  for butyrate), with the strongest reduction being observed for butyrate production, i.e. a reduction of 13.5 mM. Addition of *L. rhamnosus* GG, *S. boulardii*, or both had no major effect on SCFA production under dysbiotic conditions. Overall, similar trends in SCFA production were observed during the experiments performed with the toddler donor (Supplementary Table S1 for healthy conditions and Table S2 for dysbiotic conditions), albeit not reaching statistical

**Table 1.** Overall metabolic activity under simulated colonic healthy conditions for adult donor.<sup>1,2</sup>

	CTRL	LGG	SB	LGG + SB
Acetate (mM)	51.9 <sup>a</sup> $\pm$ 0.7	39.7 <sup>b</sup> $\pm$ 2.1	49.2 <sup>c</sup> $\pm$ 0.3	46.1 <sup>b</sup> $\pm$ 0.7
Propionate (mM)	17.6 <sup>a</sup> $\pm$ 0.4	14.8 <sup>b</sup> $\pm$ 0.8	19.7 <sup>c</sup> $\pm$ 0.3	19.2 <sup>c</sup> $\pm$ 0.4
Butyrate (mM)	17.0 <sup>a</sup> $\pm$ 0.4	25.0 <sup>b</sup> $\pm$ 0.2	12.4 <sup>c</sup> $\pm$ 0.1	17.7 <sup>d</sup> $\pm$ 0.2
Lactate (mM)	0.5 <sup>a</sup> $\pm$ 0.2	0.4 <sup>a</sup> $\pm$ 0.1	2.1 <sup>a,b</sup> $\pm$ 0.6	1.3 <sup>b</sup> $\pm$ 0.2
Ethanol (mM)	8.6 <sup>a</sup> $\pm$ 1.0	7.5 <sup>a</sup> $\pm$ 1.1	29.2 <sup>b</sup> $\pm$ 0.7	20.0 <sup>c</sup> $\pm$ 1.2
Branched SCFA (mM)	1.4 <sup>a</sup> $\pm$ 0.0	1.5 <sup>a</sup> $\pm$ 0.0	4.3 <sup>b</sup> $\pm$ 0.1	4.5 <sup>c</sup> $\pm$ 0.1
Ammonium (mg/l)	317.9 <sup>a</sup> $\pm$ 16.3	328.2 <sup>a</sup> $\pm$ 19.7	437.5 <sup>b</sup> $\pm$ 9.6	426.3 <sup>b</sup> $\pm$ 29.5

<sup>1</sup> Average change ( $\pm$  standard deviation) in acetate, propionate, butyrate, lactate, ethano, branched short chain fatty acids (SCFA) and ammonium between 0-48 h during the short-term colonic incubations using a colonic background microbiota of an adult donor ( $n=3$ ).

<sup>2</sup> *Lactobacillus rhamnosus* GG (LGG) and *Saccharomyces boulardii* (SB) were tested separately as well as together (LGG+SB) compared to a control incubation (CTRL). Statistically significant differences between experimental conditions (i.e. CTRL, LGG, SB and LGG+SB) after 48 h of incubation are highlighted by assigning different letters ( $P<0.05$ ).

**Table 2. Overall metabolic activity under simulated colonic dysbiotic conditions for adult donor.<sup>1,2</sup>**

	CTRL	LGG	SB	LGG + SB
Acetate (mM)	42.7 <sup>a</sup> ±3.2	44.3 <sup>a</sup> ±3.3	45.5 <sup>a</sup> ±7.8	42.3 <sup>a</sup> ±1.8
Propionate (mM)	13.3 <sup>a,b</sup> ±1.5	10.4 <sup>a</sup> ±1.0	13.8 <sup>a,b</sup> ±1.6	13.8 <sup>b</sup> ±0.6
Butyrate (mM)	3.5 <sup>a</sup> ±0.6	6.2 <sup>a</sup> ±0.4	3.8 <sup>a</sup> ±0.3	5.5 <sup>a</sup> ±0.2
Lactate (mM)	-0.2 <sup>a</sup> ±0.0	0.0 <sup>b</sup> ±0.0	-0.1 <sup>c</sup> ±0.0	-0.1 <sup>d</sup> ±0.0
Ethanol (mM)	11.0 <sup>a</sup> ±1.0	10.8 <sup>a</sup> ±0.7	17.9 <sup>b</sup> ±2.2	14.1 <sup>a</sup> ±0.3
Branched SCFA (mM)	1.4 <sup>a</sup> ±0.6	1.0 <sup>a</sup> ±0.0	1.7 <sup>a</sup> ±0.1	1.3 <sup>a</sup> ±0.1
Ammonium (mg/l)	265.3 <sup>a</sup> ±3.4	259.1 <sup>a,b</sup> ±14.4	276.1 <sup>a,b</sup> ±19.6	288.9 <sup>b</sup> ±2.1

<sup>1</sup> Average change (± standard deviation) in acetate, propionate, butyrate, lactate, ethanol, branched short chain fatty acids (SCFA) and ammonium between 0–48 h during the short-term colonic incubations using an adult colonic background microbiota supplemented with *Escherichia coli* ETEC (n=3).

<sup>2</sup> *Lactobacillus rhamnosus* GG (LGG) and *Saccharomyces boulardii* (SB) were tested separately as well as together (LGG+SB) compared to a control incubation (CTRL). Statistically significant differences between experimental conditions (i.e. CTRL, LGG, SB and LGG+SB) after 48 h of incubation are highlighted by assigning different letters ( $P < 0.05$ ).

significance except for a significant increase in butyrate levels upon supplementation of LGG and LGG+SB under healthy conditions.

When comparing healthy and dysbiotic conditions for the control incubations, it was observed that in the adult model lactate levels were significantly higher under healthy conditions ( $P=0.046$ ), while in the toddler model a trend towards higher lactate levels was observed under healthy conditions ( $P=0.060$ ). Upon addition of *L. rhamnosus* GG, lactate levels remained unaffected compared to the control incubations, except for a significant increase for the adult donor under dysbiotic conditions (Table 2). Addition of *S. boulardii* to the colonic incubations of the adult donor resulted in increased lactate concentrations compared to the control incubations under both healthy (Table 1) and dysbiotic (Table 2) conditions, whereas decreased lactate levels were observed for the toddler donor (Supplementary Table S1 and S2), though only reaching significance under dysbiotic conditions. Dosing of *L. rhamnosus* GG together with *S. boulardii* resulted in an averaged outcome of the effects they both generated when incubated alone.

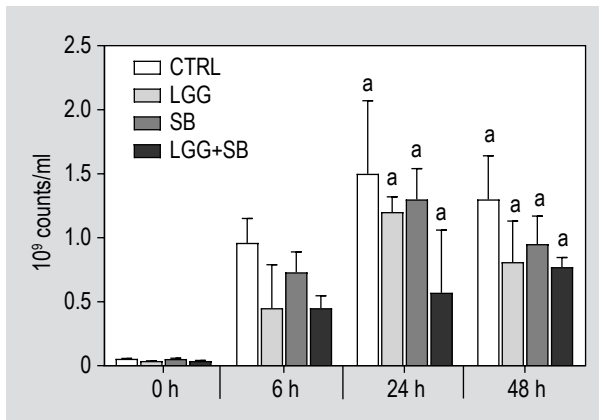
Ethanol was produced during the control experiments with both donors under healthy (Table 1 for adult and Supplementary Table S1 for toddler) and dysbiotic (Table 2 for adult and Supplementary Table S2 for toddler) conditions. Under dysbiotic conditions, ethanol concentrations were higher compared to healthy conditions for the control incubations, albeit only reaching significance

in the adult model ( $P=0.042$ ). Ethanol levels remained unaffected upon dosing of *L. rhamnosus* GG to the colonic incubations. Addition of *S. boulardii* resulted in a statistically significant increase in the production of ethanol compared to the control experiments in the adult model, whereas for the toddler donor under healthy conditions, a trend towards increased ethanol concentrations was observed. When *S. boulardii* and *L. rhamnosus* GG were administered together to the adult model, significantly increased ethanol production was observed under healthy conditions compared to the control experiments, though still significantly lower than the increase observed when *S. boulardii* was inoculated alone.

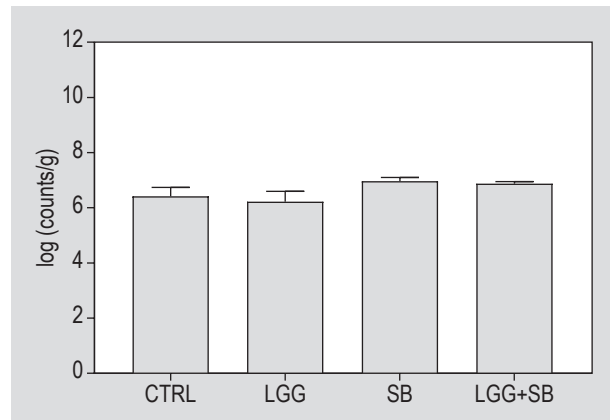
Ammonium and branched chain fatty acids (BCFA) were produced during all experiments of the present study, indicating the occurrence of proteolytic fermentation. When comparing healthy and dysbiotic conditions for the control incubations, it was observed that ammonium and BCFA levels were significantly higher under healthy conditions in both donors tested, except for BCFA levels in the adult model where similar levels were observed between both conditions. Addition of *L. rhamnosus* GG to the colonic incubations did not result in increased production of ammonium and BCFA compared to the control incubations under both healthy (Table 1 for adult and Supplementary Table S1 for toddler) and dysbiotic (Table 2 for adult and Supplementary Table S2 for toddler) conditions. Under healthy conditions, supplementation of *S. boulardii* (alone or in combination with *L. rhamnosus* GG) resulted in a statistically significant increase in ammonium and BCFA production compared to the control experiments, for both donors tested. Under dysbiotic conditions, supplementation of *L. rhamnosus* GG together with *S. boulardii* resulted in statistically significantly higher ammonium production compared to the control experiments when using an adult ( $P < 0.004$ ) and a toddler ( $P=0.050$ ) colonic background microbiota, whereas no statistically significant differences were observed for BCFA production.

### Effect of *Lactobacillus rhamnosus* GG and *Saccharomyces c. boulardii* against ETEC

Determination of the growth of ETEC during the control incubations demonstrated that this pathogenic strain was capable to outgrow in the presence of the dysbiotic colonic background community of both the adult (Figure 2) and toddler (Supplementary Figure S2) donor. Indeed, during both sets of experiments, ETEC reached high cell abundances after 24 h of incubation. Administration of *S. boulardii* to the experiments had no major effect on the growth of ETEC. On the contrary, supplementation of *L. rhamnosus* GG resulted in lower concentrations of ETEC throughout the course of the experiments, with the strongest effect being observed after 24 h, when both *L. rhamnosus* GG and *S. boulardii* were added to the



**Figure 2.** Growth of *Escherichia coli* ETEC in microbiota colonic incubations from an adult donor. Mean counts/ml ( $\pm$  standard deviation) of ETEC in the lumen at several time points during the short-term colonic incubations using a colonic background microbiota of an adult donor supplemented with ETEC (n=3). *Lactobacillus rhamnosus* GG (LGG) and *Saccharomyces boulardii* (SB) were tested separately as well as together (LGG+SB) compared to a control incubation (CTRL). Statistically significant differences between experimental conditions (i.e. CTRL, LGG, SB and LGG+SB) after 24 and 48 h of incubation are highlighted by assigning different letters ( $P < 0.05$ ).



**Figure 3.** Growth of mucosal *Escherichia coli* ETEC in microbiota colonic incubations from an adult donor. Mean counts/ml ( $\pm$  standard deviation) of ETEC in the mucus after 48 h during the short-term colonic incubations using a colonic background microbiota of an adult donor supplemented with ETEC (n=3). *Lactobacillus rhamnosus* GG (LGG) and *Saccharomyces boulardii* (SB) were tested separately as well as together (LGG+SB) compared to a control incubation (CTRL). Statistically significant differences between experimental conditions (i.e. CTRL, LGG, SB and LGG+SB) after 48 h of incubation are highlighted by assigning different letters ( $P < 0.05$ ).

incubations. A 40 and 46% reduction in the concentration of ETEC was observed upon addition of both strains during the experiments with the adult donor and toddler donor, respectively, reaching statistical significance during the experiments using the toddler colonic background community ( $P = 0.013$ ).

ETEC was able to adhere to the mucus beads for both the adult (Figure 3) and the toddler (Supplementary Figure S3) donor, resulting in an average adhesion of 6.6 and 7.0 log cfu/g, respectively. *L. rhamnosus* GG and *S. boulardii* as well as their combination did not reduce ETEC binding for both donors.

The results obtained during the agar well diffusion experiments (Table 3) indicated that the culture supernatant of *L. rhamnosus* GG had an antimicrobial effect on ETEC. This was mainly due to the low pH of the supernatant (pH=4.4) since no inhibition zones were obtained when the culture supernatant was adjusted to pH 6.5. Large inhibition zones were obtained when the sonicated cell suspensions of *L. rhamnosus* GG were administered to the plates. Finally, metabolically active cells of *L. rhamnosus* GG also resulted in the occurrence of inhibition zones. The culture supernatant of *S. boulardii* had no antimicrobial effect against ETEC. On the contrary, the sonicated fraction of the *S. boulardii* culture resulted in inhibition zones through its antimicrobial activity against ETEC. Finally, also the metabolically active *S. boulardii* cells resulted in inhibition zones.

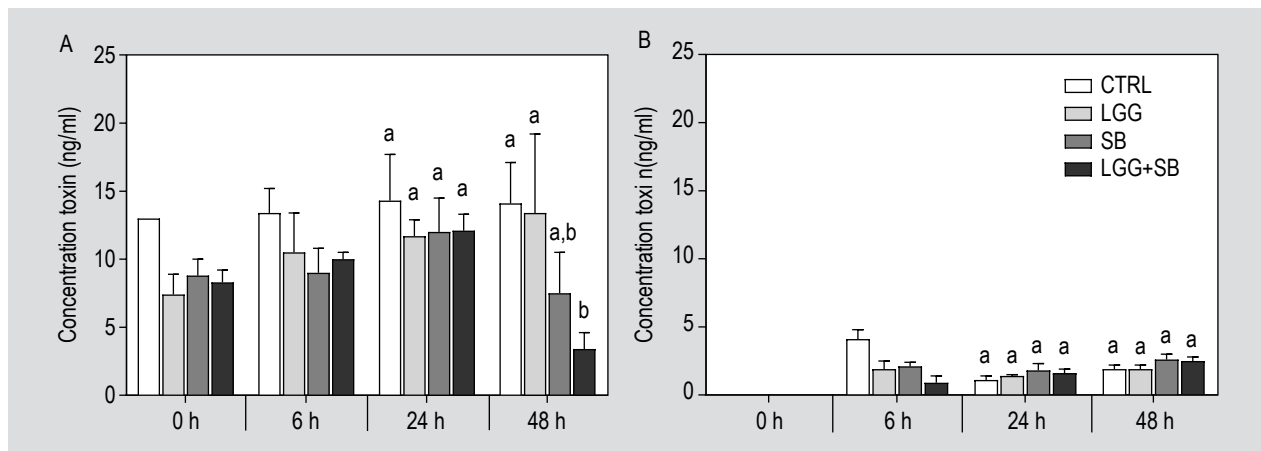
Determination of the concentration of toxin produced in the supernatant revealed that the growth of ETEC resulted in the production of its toxin over the course of the control incubations and this for both donors (Figure 4 for adult and Supplementary Figure S5 for toddler). Administration of *L. rhamnosus* GG to the incubations had no effect on the production of the toxin by ETEC in the supernatant. On the

**Table 3.** Agar well diffusion assay.<sup>1</sup>

Probiotic	Fraction	Agar well diffusion <sup>2</sup>
<i>Lactobacillus rhamnosus</i> GG	Supernatant	2 mm inhibition zone
	Supernatant; neutral pH	No inhibition zone
	Sonicated	4 mm inhibition zone <sup>2</sup>
	Live cells	3 mm inhibition zone <sup>2</sup>
<i>Saccharomyces boulardii</i>	Supernatant	No inhibition zone
	Supernatant; neutral pH	No inhibition zone
	Sonicated	2 mm inhibition zone <sup>2</sup>
	Live cells	1 mm inhibition zone <sup>2</sup>

<sup>1</sup> Antimicrobial activity of different *Lactobacillus rhamnosus* GG and *Saccharomyces boulardii* fractions (i.e. supernatant, supernatant at neutral pH, sonicated cells and live cells) against *Escherichia coli* ETEC as determined by measuring the inhibition zone in the agar well diffusion assay.

<sup>2</sup> Shown in Supplementary Figure S4.



**Figure 4.** *Escherichia coli* ETEC toxins in a colonic microbiota of an adult donor. Average concentration (ng/ml) ( $\pm$  standard deviation) of ETEC toxin present in (A) the supernatant and (B) bound to the bacterial cells (pellet) at several time points during the short-term colonic incubations using a colonic background microbiota of an adult donor supplemented with ETEC ( $n=3$ ). *Lactobacillus rhamnosus* GG (LGG) and *Saccharomyces boulardii* (SB) were tested separately as well as together (LGG+SB) compared to a control incubation (CTRL). Statistically significant differences between experimental conditions (i.e. CTRL, LGG, SB and LGG+SB) after 24 and 48 h of incubation are highlighted by assigning different letters ( $P<0.05$ ).

contrary, lower concentrations of toxin were present in the supernatant after 48 h of incubation during the experiments with *S. boulardii*, albeit not statistically significant, and this for both donors. During the experiments to which both *L. rhamnosus* GG and *S. boulardii* were supplemented, ETEC was capable to produce its toxin during the first 6 and 24 h of incubation resulting in elevated toxin levels in the supernatant. The concentration of toxin subsequently decreased between 24 and 48 h of incubation to a significantly lower concentration compared to the control incubations and this both for the adult ( $P=0.015$ ) and toddler ( $P=0.006$ ) donor. The concentration of cell wall-bound toxin was lower compared to the concentration in the supernatant and this for all different sets of experiments. Administration of *L. rhamnosus* GG, *S. boulardii*, or *L. rhamnosus* GG together with *S. boulardii* had no effect on the concentration of toxin that was bound to the cell wall. The sum of the concentrations of the toxin in the supernatant and bound to the cell wall showed that *L. rhamnosus* GG and *S. boulardii* had a synergistic effect against toxin production by ETEC and this both for the adult donor ( $P=0.018$ ) and for the toddler ( $P=0.055$ ) donor, resulting in a reduction of 57.4 and 45.5%, respectively.

#### 4. Discussion

To our knowledge, the well-documented strains *L. rhamnosus* GG and *S. boulardii* have never been studied together before. As both strains confer several health benefits, we aimed to study the interaction between *L. rhamnosus* GG and *S. boulardii* at concentrations which are in the range of usual therapeutic doses in humans, their modulatory effect on the colonic microbial functionality and the possible synergistic effects when both strains are

added together into a healthy colonic environment using an *in vitro* model. Two different donors were used as a source of a colonic background community, and this to account for inter-individual differences. As the microbiome in toddlers is similar to that in adults (Voreades *et al.*, 2014), both types of donors were selected for the current study.

Addition of *L. rhamnosus* GG to the colonic incubations resulted in a high concentration of this strain at the start of the experiments, followed by minor growth throughout the incubation period. Furthermore, determination of *L. rhamnosus* GG concentrations present on the mucin-coated beads revealed that this strain was capable to adhere to the mucosal layer of the simulated colon. Indeed, Kankainen *et al.* (2009) revealed that the surface of *L. rhamnosus* GG contains mucus-binding pili, which shows its ability to bind to the mucosal layer in the colon. Co-cultivation of *L. rhamnosus* GG together with *S. boulardii* resulted in comparable concentrations of *L. rhamnosus* GG in both the luminal and mucosal compartments of the incubations, which indicated that the activity of *S. boulardii* had no negative impact on *L. rhamnosus* GG in the lumen and that *S. boulardii* did not compete with *L. rhamnosus* GG for adhesion sites in the mucosa. Dosing of *S. boulardii* to the incubations resulted in high concentrations of this strain during the first 6 h of the experiments. Afterwards, levels of *S. boulardii* decreased and this especially after 48 h. This was probably due to competition for substrates with the colonic background microbiota and not due to competition with *L. rhamnosus* GG, as this occurred when *S. boulardii* was grown alone and similar effects were observed when both strains were added to the incubations. In the adult model, even significantly higher concentrations of *S. boulardii* were observed after 24 and 48h of incubation



compared to the incubations that were performed with *S. boulardii* alone. This could indicate a protective effect of *L. rhamnosus* GG towards *S. boulardii*. Furthermore, *S. boulardii* was also capable of binding the mucin-coated beads present in the incubations and its adhesion was not affected by the presence of *L. rhamnosus* GG. Adhesion was lower compared with *L. rhamnosus* GG, which could be explained by the absence of mucus-binding pili for *S. boulardii* (Moré and Swidsinski, 2015).

Determination of the metabolites produced during these experiments revealed the additive effect of *L. rhamnosus* GG and *S. boulardii* when inoculated together to the colon under healthy conditions. Dosing of *L. rhamnosus* GG to the colonic microbiota resulted in the production of increased levels of butyrate compared to the control. Butyrate is a major energy source for colonocytes (Basson *et al.*, 1996) and has been associated with several health-promoting properties, such as reduction of oxidative stress, improvement of gut barrier function and enhancement of anti-inflammatory response (Hamer *et al.*, 2008). As one of the main end metabolites of *L. rhamnosus* GG fermentation is lactate (De Keersmaecker *et al.*, 2006), the observed increase in butyrate levels was probably attributed to stimulation of specific lactate-utilising, butyrate-producing bacterial species such as *Anaerostipes caccae* and *Eubacterium hallii* (Duncan *et al.*, 2004). The utilisation of lactate by these microorganisms probably resulted in an absence of increase on lactate levels during the incubation period due to production and subsequent consumption of this transient metabolite (Moens *et al.*, 2017). Furthermore, during conversion of lactate into butyrate, acetate is an essential co-substrate that needs to be consumed to complete the final reaction step in butyrate synthesis (Duncan *et al.*, 2002), which was indeed observed by a reduction of acetate levels upon dosing *L. rhamnosus* GG to the experiments. Dosing of *S. boulardii* gave rise to the production of high concentrations of ethanol. Ethanol is known for its antimicrobial properties but is in general not a major end-metabolite produced during colonic fermentation processes. Therefore, ethanol production can mainly be attributed to the fermentative activity of *S. boulardii* during the aforementioned incubations. Furthermore, addition of *S. boulardii* to the colonic microbiota resulted in increased production of propionate throughout the course of the incubation, which could be linked to the decreased concentrations of luminal *S. boulardii* after 48 h of incubation. Indeed, the decreased *S. boulardii* concentrations suggested the occurrence of cell lysis resulting in the release of major cell wall components in the medium, which could be used as substrates by propionate-producing bacteria. Cuskin *et al.* (2015) reported that the propionate-producing *Bacteroidetes* species can utilise mannan-oligosaccharides, which are present in the outer cell wall of yeast. Hughes *et al.* (2008) reported that beta-glucans, another major cell

wall component of yeasts (Manners *et al.*, 1973), specifically stimulate propionate during colonic fermentation. Co-cultivation of both *L. rhamnosus* GG and *S. boulardii* resulted in increased butyrate, propionate, and ethanol production compared to the control incubations indicating the cooperative effect of both strains.

Moreover, the antipathogenic effect of *L. rhamnosus* GG and *S. boulardii* against ETEC was investigated in a colonic dysbiotic environment. Dysbiosis was confirmed by the significantly decreased levels of SCFA and ammonium compared to healthy conditions. ETEC was capable to outgrow in the dysbiotic microbiota of both donors and to adhere to the mucosal surface. Addition of *L. rhamnosus* GG to the incubations resulted in lower concentrations of luminal ETEC at each sampling point, with the lowest concentration of ETEC observed when *L. rhamnosus* GG and *S. boulardii* were dosed together. The antimicrobial activity of *L. rhamnosus* GG was mainly attributed to the active production of toxic metabolites as large inhibition zones were obtained during the agar well diffusion assay in the presence of the culture supernatant at low environmental pH. The antimicrobial activity of *L. rhamnosus* GG was probably attributed to the production of lactate, as was already reported by De Keersmaecker *et al.* (2006). Lactate decreases the pH of the environment (Macfarlane and Englyst, 1986). Especially at low pH values, lactate can exert strong antimicrobial effects against pathogens, as protonated lactic acid can penetrate the microbial cell after which it dissociates and releases protons within the cell, resulting in acidification and microbial cell death (Alakomi *et al.*, 2000; Raybaudi-Massilia *et al.*, 2009; Stratford and Eklund, 2003). Therefore, the antimicrobial activity of *L. rhamnosus* GG could be attributed to lactate production under acidic environmental conditions. Administration of *S. boulardii* also resulted in antimicrobial activity against ETEC in the luminal environment. The sonicated fraction of the *S. boulardii* culture resulted in the largest inhibition zones, indicating that cell components of *S. boulardii* were toxic for ETEC. Additionally, dosing of *S. boulardii* to the experiments resulted in lower toxin concentrations after 48 h of incubation compared to the control experiments, with an increased effect being observed when *L. rhamnosus* GG and *S. boulardii* were co-cultivated. The effect on toxin production by *S. boulardii* could be explained by the secretion of proteolytic enzymes. Indeed, the production of a specific protease by *S. boulardii* has already been shown to inhibit the effects of *Clostridium difficile* toxins A and B in human colonic mucosa (Castagliuolo *et al.*, 1999). Also, Buts *et al.* (2006) reported that *S. boulardii* produces a protein phosphatase that inhibited *E. coli* endotoxins in the small intestine of rats. Furthermore, Roussel *et al.* (2018) demonstrated that the probiotic yeast *Saccharomyces cerevisiae* CNCM I-3856 reduces ETEC toxin concentrations in culture media through active removal of the toxin by the yeast strain. The increased

ammonium and branched SCFA concentrations observed during the incubations with *S. boulardii* confirms the stimulation of proteolytic activity. Hence, addition of *L. rhamnosus* GG together with *S. boulardii* resulted in a stronger effect which was probably due to the fact that the growth of *L. rhamnosus* GG resulted in a faster and more pronounced depletion of carbohydrates thereby increasing the proteolytic activity of *S. boulardii* towards the toxin. This also explains why the toxin concentration initially increased during the experiments with *S. boulardii* to comparable concentrations as the control, as during the initial 24 h of the incubation carbohydrates were still present in the medium which stimulated saccharolytic fermentation by *S. boulardii* thereby minimising its proteolytic activity. While all antimicrobial activity against ETEC was observed in the luminal environment, both *L. rhamnosus* GG and *S. boulardii* were not capable to compete for binding sites with ETEC on the mucosa. However, the mucus layer generally creates a protective barrier (Cornick *et al.*, 2015) and therefore one single dose of the test strains in the current experimental setup was probably not effective to observe antipathogenic effects in the mucosal environment. Whereas, *L. rhamnosus* GG and *S. boulardii* had a detrimental effect on ETEC in the lumen, they did not seem to exert these effects on the residing microbial community of both donors, as was seen by the stable SCFA levels compared to the control under these dysbiotic conditions. The activity of the strains even resulted in the production of additional butyrate (by *L. rhamnosus* GG) and ethanol (by *S. boulardii*) during the colonic incubations. Altogether, these results demonstrated the complementarity of the antipathogenic effects of these strains on ETEC, resulting in a significantly lower amount of ETEC toxin after 48 h.

Some potential shortcomings of the study design include the absence of analysis of the microbial community composition. Indeed, in the current study, impact of *L. rhamnosus* GG and *S. boulardii* on the residing microbiota under both healthy and dysbiotic conditions was mainly investigated through assessment of microbial functionality. Analysis of microbial community composition might have provided further insight into the effect of both probiotic strains on the residing microbial community. Indeed, presence of specific microbial groups in the microbial community of the respective donors might have contributed to the observed synergistic effects and therefore investigation of the potential correlations between the observed effects and the initial microbial community composition might provide insight into the responsiveness of subjects to the probiotic formulation. Furthermore, the effect of both strains was examined upon administration of one single dose, while *in vivo* probiotics are generally consumed over several days or weeks. Inclusion of the long-term evaluation of repeated probiotic intake might have provided further insight on the effect of *L. rhamnosus* GG, *S. boulardii* and

their combination against ETEC. Finally, in the current experimental setting, a dysbiotic microbial community was simulated by the administration of very low percentage of the background microbial community of the donors under investigation. The strength of this approach lies in the fact that the impact of inter-individual variation was minimised allowing for physiologically relevant comparisons between both healthy and impaired conditions. However, while dilution of the faecal inoculum will have resulted in a dysbiotic microbial community in a quantitative way, the qualitative microbiota composition might not be as accurate as would be observed *in vivo*.

In conclusion, the results indicated that *L. rhamnosus* GG and *S. boulardii* were capable to grow together and no antagonistic effects were observed between both strains. Co-cultivation showed or resulted in the complementary of both strains at the metabolic level, which potentiated the antipathogenic and antitoxin activity against ETEC. Altogether, the results reinforce the hypothesis that both probiotics together may help microbiota functionality, either in adults or toddlers and under healthy or impaired conditions. This effect could be of great interest under dysbiotic conditions such as during antibiotic therapy, when the colonic microbiota is more sensitive to invasion of pathogens. Future research is warranted to further explore this hypothesis.

## Supplementary material

Supplementary material can be found online at <https://doi.org/10.3920/BM2019.0064>.

**Figure S1.** Growth of *Lactobacillus rhamnosus* GG and *Saccharomyces boulardii* in microbiota colonic incubations from a toddler donor.

**Figure S2.** Growth of *Escherichia coli* ETEC in microbiota colonic incubations from a toddler donor.

**Figure S3.** Growth of mucosal *Escherichia coli* ETEC in microbiota colonic incubations from a toddler donor.

**Figure S4.** Agar well diffusion assay.

**Figure S5.** *Escherichia coli* ETEC toxins in a colonic microbiota of a toddler donor.

**Table S1.** Overall metabolic activity under simulated colonic healthy conditions for toddler donor.

**Table S2.** Overall metabolic activity under simulated colonic dysbiosed conditions for toddler donor.

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## Conflict of interest

Mireia Morera is an employee of Ipsen Pharma SAS.

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