Unexpected consequences of administering bacteriocinogenic probiotic strains for *Salmonella* populations, revealed by an *in vitro* colonic model of the child gut

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New biological strategies for the treatment of *Salmonella* infection are needed in response to the increase in antibiotic-resistant strains. *Escherichia coli* L1000 and *Bifidobacterium thermophilum* RBL67 were previously shown to produce antimicrobial proteinaceous compounds (microcin B17 and thermophilicin B67, respectively) active *in vitro* against a panel of *Salmonella* strains recently isolated from clinical cases in Switzerland. In this study, two three-stage intestinal continuous fermentation models of *Salmonella* colonization inoculated with immobilized faeces of a two-year-old child were implemented to study the effects of the two bacteriocinogenic strains compared with a bacteriocin-negative mutant of strain L1000 on *Salmonella* growth, as well as gut microbiota composition and metabolic activity. Immobilized *E. coli* L1000 added to the proximal colon reactor showed a low colonization, and developed preferentially in the distal colon reactor independent of the presence of genetic determinants for microcin B17 production. Surprisingly, *E. coli* L1000 addition strongly stimulated *Salmonella* growth in all three reactors. In contrast, *B. thermophilum* RBL67 added in a second phase stabilized at high levels in all reactors, but could not inhibit *Salmonella* already present at a high level ($>10^7$ c.f.u. ml⁻¹) when the probiotic was added. Inulin added at the end of fermentation induced a strong bifidogenic effect in all three colon reactors and a significant increase of *Salmonella* counts in the distal colon reactor. Our data show that under the simulated child colonic conditions, the microcin B17 production phenotype does not correlate with inhibition of *Salmonella* but leads to a better colonization of *E. coli* L1000 in the distal colon reactor. We conclude that *in vitro* models with complex and complete gut microbiota are required to accurately assess the potential and efficacy of probiotics with respect to *Salmonella* colonization in the gut.

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INTRODUCTION

The human gastrointestinal tract is colonized by a complex community of micro-organisms that plays a key protective role during enteric infections (Stecher & Hardt, 2008). Infectious gastroenteritis caused by non-typhoidal strains of *Salmonella enterica* subsp. *enterica* is an important cause of morbidity and mortality worldwide, especially in young children (Crum-Cianflone, 2008; Viswanathan *et al.*, 2009). Due to an increasing incidence of antibiotic-resistant and more infective serovars (Kingsley *et al.*, 2009), the use of probiotics with specific anti-*Salmonella* activities is of current interest. Mechanisms by which probiotics inhibit

pathogens include competition for nutritional substrates and adhesion sites on intestinal epithelial cells, toxin inactivation, as well as the secretion of antimicrobial substances (Collado *et al.*, 2009).

Bacteriocins are promising candidates as mediators of anti-pathogenic probiotic effects, and their ability to inhibit pathogens *in vitro* is well documented (Corr *et al.*, 2009). Corr *et al.* (2007), for example, showed that *Lactobacillus salivarius* UCC118 is able to produce the bacteriocin Abp118 *in vivo*, resulting in the protection of mice against *Listeria monocytogenes* infection. Using an ICR mouse model infected with *Listeria*, Dabour *et al.* (2009) recently showed that intragastric administration of repeated doses of pediocin PA-1 reduces *Listeria* counts and pathogen translocation into the liver and spleen compared with the

Abbreviations: MCN, mean copy number; real-time qPCR, real-time quantitative PCR; SCFA, short-chain fatty acid.

infected control group, leading to the disappearance of *L. monocytogenes* in these two organs within 6 days. However, enteric protection related to *in situ* bacteriocin production in human gut environments has been little studied.

Using simple *in vitro* activity tests, we recently showed that *Escherichia coli* L1000 producing microcin B17 is active against a broad panel of antibiotic-resistant and -sensitive *Salmonella* strains isolated in Switzerland from patients suffering from salmonellosis (Zihler *et al.*, 2009). Furthermore, thermophilicin B67, a bacteriocin-like inhibitory substance produced by the Gram-positive bacterium *Bifidobacterium thermophilum* RBL67 (von Ah, 2006; von Ah *et al.*, 2007), exerts strong inhibitory activity against 10 strains of the same panel belonging to six different serovars (our unpublished data). Although simple *in vitro* antagonist tests are useful for screening new probiotic bacteria with specific antimicrobial activity, gut environments are much more complex. Dynamic interactions between microorganisms are responsible for colonization and competition, and in maintaining a healthy balance and the barrier effect of the gut microbiota *in vivo*. More elaborate models involving a complex gut microbiota providing conditions closer to those found in gastrointestinal environments are therefore required for activity and mechanistic studies of potential probiotics before considering more complex and expensive *in vivo* testing.

A single-stage continuous fermentation model for *Salmonella* colonization in the proximal colon that mimics long-term shedding in children was recently developed and used to compare the effects of antibiotic therapy and *B. thermophilum* RBL67 on salmonellosis in child gut environments (Le Blay *et al.*, 2009; A. Zihler and others, unpublished results). *B. thermophilum* RBL67 grows in the intestinal environment and exhibits a strong inhibition of *Salmonella* when added before (preventive) or after (curative) *Salmonella*, and is able to rebalance the metabolic activity of gut microbiota after antibiotic treatments. These experiments were performed with *S. enterica* subsp. *enterica* serovar Typhimurium (*S. Typhimurium*) M557, a strain lacking *Salmonella* pathogenicity island (SPI)-1 effector proteins, and deficient in SPI-1 and SPI-2-dependent type III protein secretion systems that are known to be important host-microbe virulence factors of *Salmonella* (Hapfelmeier *et al.*, 2004; Ohl & Miller, 2001).

In the present study, we tested the effects of a combined treatment with *E. coli* L1000 followed by *B. thermophilum* RBL67 on *Salmonella* growth, as well as gut microbiota composition and metabolic activity, using three-stage *in vitro* continuous gut fermentation models (Cinquin *et al.*, 2006) that mimic the infected child colon, employing *S. Typhimurium* N-15, a highly invasive strain that was isolated from a clinical case (Zihler *et al.*, 2009). *E. coli* L1000 was first tested by inoculating *E. coli* L1000 beads into the first reactor to model a colonizing *E. coli* strain, while the colonization and protective effect of *E. coli* L1000 was assumed to occur preferentially in the distal colon reactor, close to neutral pH. *B. thermophilum* RBL67 was

inoculated in a second step to preferentially induce a protective effect in the proximal colon reactor at the lower pH of 5.7. Two three-stage models were operated in parallel to test comparatively the effects of *E. coli* L1000 wild-type (*wt*) producing microcin B17 and the bacteriocin-negative mutant *E. coli* L1000 *MccB17*[−]. Finally, a prebiotic treatment with inulin was tested to stimulate *B. thermophilum* RBL67 growth.

METHODS

Bacterial strains. *S. Typhimurium* N-15 was isolated in 2007 from an infected person in Switzerland and obtained from the National Center for Enteropathogenic Bacteria (NENT; Luzern, Switzerland). This strain was selected for its high invasion rate into mucus-secreting intestinal HT29-MTX cells compared with other *Salmonella* strains obtained from clinical cases, accompanied by a high disruption of epithelial integrity as revealed by transepithelial electrical resistance (TER) measurements (our unpublished data). It was routinely cultivated in tryptic soy broth (TSB; Difco) at 37 °C for 18 h.

E. coli L1000 *wt* was provided by Laves-Arzneimittel. *E. coli* L1000 *MccB17*[−], lacking the microcin B17 phenotype, was constructed as described below. *E. coli* NCTC 50154, obtained from the UK National Collection of Type Cultures, was used as a microcin B17-sensitive indicator organism. *E. coli* strains were routinely grown on Luria-Bertani agar (LB; Becton Dickinson) for 18 h at 37 °C.

B. thermophilum RBL67 (our culture collection) was routinely grown at 37 °C for 18 h anaerobically in de Man Rogosa Sharpe broth supplemented with 0.05 % (w/v) L-cysteine HCl (MRSC; Sigma-Aldrich) in anaerobic jars with the AnaeroGen atmosphere generation system (Oxoid).

Mutant construction. The operon *mcbABCDEFG*, encoding microcin B17 production and immunity, was chosen as a target for gene deletion using the Quick & Easy *E. coli* Gene Deletion kit from Gene Bridges (BioCat) according to the manufacturer's protocol. First, a PCR product of the FRT-PGK-gb2-neo-FRT cassette containing the flanking regions of *mcbABCDEFG* was amplified using the primers *mcbA* upper and *mcbG* lower (Table 1). The resulting 1737 bp product contained the FRT-PGK-gb2-neo-FRT cassette flanked by 50 bp up- and downstream regions of *mcbABCDEFG* introduced in the primers. The strain *E. coli* L1000 *wt* was transformed by electroporation with plasmid pRedET carrying the genes for recombination, resulting in strain *E. coli* L1000-pRedET. The linear FRT-PGK-gb2-neo-FRT-*mcbABCDEFG* fragment was transformed by electroporation into *E. coli* L1000-pRedET and after induction of recombination, primary integrants were selected on LB agar containing 15 µg kanamycin ml^{−1}. Integrants were checked for site-specific integration by PCR using the primer pairs *mcbA* upper-control/Primer 2 (Table 1), annealing in the upstream and cassette promoter regions, and primer set Primer 3/*mcbG* lower-control, annealing in the downstream region and the kanamycin-resistance gene. The strain L1001, displaying the correct PCR profile, was cured according to the manufacturer's protocol, resulting in the *mcbABCDEFG*-deletion strain *E. coli* L1000 *MccB17*[−]. The mutant was checked for a microcin B17-negative phenotype using a modified protocol of the double-agar layer assay described by Kheadr *et al.* (2010). Briefly, 2 µl of overnight cultures of *E. coli* L1000 *wt* and *E. coli* L1000 *MccB17*[−] were spotted onto the surface of LB agar (1.5 %, w/v) and plates were left to dry aseptically for 30 min. After incubation at 37 °C for 18 h, colonies were treated with 10 µl chloroform (Sigma-Aldrich) for 1 min in closed Petri dishes and subsequently dried in opened dishes. A second layer of 15 ml LB agar (0.8 %, w/v) seeded with 1 % (v/v) of an

Table 1. Primers and probes used for the construction of the mutant *E. coli* L1000 *MccB17*– and the detection of different bacterial groups in faecal microbiota by real-time qPCR analysis

Target	Primer or probe	Sequence (5'–3')	Source or reference
Mutant construction			
Upstream of <i>mcbA</i> (underlined)	mcbA upper	ACG GCA AGT AAC TAG TGT TGG CCA ACA TAC TAT TCA GAT GTC ATA AGC ATA ATT AAC CCT CAC TAA AGG GCG	This study
Downstream of <i>mcbG</i> (underlined)	mcbG lower	ATC GAG ATG GAG AAC TCA GTC AAT GTT CCA TTC TTT CTG AAA TAT GTG TGT AAT ACG ACT CAC TAT AGG GCT C	This study
Upstream of the 5' integration site of <i>mcbABCDEFG</i>	mcbA upper-control	CAG GCG TAC AAA TTT AGT TC	This study
Downstream of the 3' integration site of <i>mcbABCDEFG</i>	mcbG lower-control	CTC AAA ATG CTG GGA TAC CC	This study
Promoter region of the cassette (reverse)	Primer 2	CGA GAC TAG TGA GAC GTG CTA C	*
Kanamycin-resistance gene of the cassette (forward)	Primer 3	TAT CAG GAC ATA GCG TTG GCT ACC	*
Real-time qPCR			
All bacteria	Eub338F	ACT CCT ACG GGA GGC AGC AG	Guo <i>et al.</i> (2008)
	Eub518R	ATT ACC GCG GCT GCT GG	
<i>Bacteroides</i> spp.	Bac303F	GAA GGT CCC CCA CAT TG	Ramirez-Farias <i>et al.</i> (2009)
	Bfr-Fmrev	CGC KAC TTG GCT GGT TCA G	
<i>Bifidobacterium</i> spp.	xfp-fw	ATC TTC GGA CCB GAY GAG AC	Cleusix <i>et al.</i> (2010)
	xfp-rv	CGA TVA CGT GVA CGA AGG AC	
<i>B. thermophilum</i>	bthermRTF	TTG CTT GCG GGT GAG AGT	Mathys <i>et al.</i> (2008)
	bthermRTR	CGC CAA CAA GCT GAT AGG AC	
	bthermTqM	†FAM-ATG TGC CGG GCT CCT GCA T-†TAMRA	
<i>Enterobacteriaceae</i>	Eco1457F	CAT TGA CGT TAC CCG CAG AAG AAG C	Bartosch <i>et al.</i> (2004)
	Eco1652R	CTC TAC GAG ACT CAA GCT TGC	
<i>E. coli</i> L1000 wt	mcbAF	CAC GCC AGT CTC CAT TAG GT	This study
	mcbAR	CCG TTT CCA CCA CTA CAA CC	
<i>E. coli</i> L1000 <i>MccB17</i> –	mcbAFMut	TTG GCC AAC ATA CTA TTC AGA TG	This study
	mcbARMut	GAC TCA CTA TAG GGC TCG AGG A	
<i>Lactobacillus/Leuconostoc/Pediococcus</i> spp.	F_Lacto 05	AGC AGT AGG GAA TCT TCC A	Furet <i>et al.</i> (2009)
	R_Lacto 04	CGC CAC TGG TGT TCY TCC ATA TA	

*Primers were provided within the Quick & Easy *E. coli* Gene Deletion kit (Gene Bridges GmbH).

†FAM, 6-carboxyfluorescein (fluorescent reporter dye); TAMRA, 6-carboxytetramethylrhodamine (quencher).

overnight culture of the indicator strain *E. coli* NCTC 50154 or *S. Typhimurium* N-15 was poured on the plates. After agar solidification, plates were incubated at 37 °C for 18 h and checked for the absence of inhibition zones.

Bacterial immobilization. A faecal sample (~5 g) from a healthy 2-year-old child (male), receiving a fully diversified diet and not exposed to antibiotic therapy for at least 3 months before the experiment, was collected. The sample was transferred to a tube containing 5 ml sterile, pre-reduced peptone water (0.1 %, pH 7), placed in an anaerobic jar, and immediately delivered to the laboratory before being homogenized. The faecal suspension was weighed and the volume adjusted with peptone water to obtain a final faecal concentration of 20 % (w/v). The faecal bacterial extract was immobilized in 1–2 mm diameter gel beads composed of 2.5 % gellan gum, 0.25 % xanthan gum and 0.2 % sodium citrate (w/v, Sigma-Aldrich), as described previously (Cleusix *et al.*, 2008; Le Blay *et al.*, 2009). The entire process was performed under anaerobic conditions within 3 h after defecation. An inoculum of 60 ml (30 %, v/v) of fresh faecal beads was immediately transferred to the first reactors of both three-stage fermentation systems.

Ten-fold-concentrated overnight cultures of *S. Typhimurium* N-15, *E. coli* L1000 *wt*, *E. coli* L1000 *MccB17*– and *B. thermophilum* RBL67 were separately immobilized in the same way but aerobically. Bead colonization was performed with batch cultures in TSB for *Salmonella* (18 h, 37 °C, no pH control), in LB for *E. coli* (18 h, 37 °C, no pH control) and in MRSC for *B. thermophilum* RBL67 (24 h, 40 °C, pH 6.0, stirring at 150 r.p.m.). Colonized beads were washed in sterile CaCl₂ (0.1 mol l⁻¹) and used to inoculate the first or third reactor of the appropriate fermentation system, as described below.

Nutritive culture medium. A complex culture medium simulating the intestinal chyme of a young child was used to feed the first reactor of each three-stage fermentation system. The medium was similar to that described by Macfarlane *et al.* (1998) and modified for children by reducing the bile salt concentration from 0.4 to 0.05 g l⁻¹ in order to reproduce the ileal chyme of a young child (Le Blay *et al.*, 2009). A 0.5 ml volume of a filter-sterilized (Minisart, 0.2 µm pore-size, Sartorius) vitamin solution (Michel *et al.*, 1998) was added to 1 l of the autoclaved (15 min, 121 °C) medium. For prebiotic treatments, the medium was supplemented with 15 g l⁻¹ high-solubility inulin (Orafti HIS, Beneo Orafti) before autoclaving.

Fermentation procedures. Faecal bead colonization was performed during batch fermentations for 48 h in two reactors (Sixfors, Infors) containing 30 % fresh beads and operated at a working volume of

200 ml at pH 5.7 and 37 °C, as previously described (Le Blay *et al.*, 2009). The fermented medium was replaced every 12 h with fresh nutritive medium. Then, two additional reactors (R2 and R3), half-filled with sterile nutritive medium, were connected in series to the first reactor, R1. Continuous fermentation was started by connecting a stirred feedstock vessel containing sterile nutritive medium (4 °C) via peristaltic pumps (Reglo Analog, Ismatec) to R1. Fermented medium from R1 was then pumped to R2 (working volume 400 ml), from R2 to R3 (working volume 400 ml), and finally from R3 to an effluent receiving vessel. The retention times and pH in R1, R2 and R3 were selected to mimic *in vivo* conditions in the child proximal, transverse and distal colons (Fallingborg *et al.*, 1990; Wagener *et al.*, 2004). The total mean retention time of the system with a total volume of 800 ml was set at 25 h by adjusting the feed flow rate to 40 ml h⁻¹, resulting in mean retention times of 5 h for R1 and 10 h for R2 and R3. The headspaces of the reactors were continuously flushed with CO₂ to maintain anaerobic conditions. The pH in R1, R2 and R3 was automatically controlled at pH 5.7, 6.2 and 6.7, respectively, by adding 5 M NaOH.

The two three-stage reactor models (F1 and F2) were operated in parallel for a total of 65 days, which was divided into different periods, as shown in Fig. 1. After bead colonization and stabilization for 10 days (Stab), *S. Typhimurium* N-15 beads (2.3×10^9 c.f.u. g⁻¹) were added twice to the first reactor (R1) of both models (2 g on day 13 and 2.5 g on day 17) to induce *Salmonella* colonization. Continuous culture was carried out until day 22 (Sal). Beads containing the microcin B17-producing wild-type strain of *E. coli* (L1000 *wt*) were added on day 22 to R1 of F1 (5 g, 1.5×10^9 c.f.u. g⁻¹, Ecol I) and repeated on day 36 in R3 (5 g, 1.4×10^9 c.f.u. g⁻¹, Ecol II). For F2, the same protocol was applied but the wild-type *E. coli* strain was replaced by the mutant *E. coli* L1000 *MccB17*–, which did not produce microcin B17. On day 44, 5 g *B. thermophilum* RBL67 beads (3.4×10^{10} c.f.u. g⁻¹) were added to R1 of both models (F1, Bif I; F2, Bif). This treatment was repeated on day 56 in the first fermentation model (F1, Bif II), while in the second fermentation model (F2), the nutritive medium was supplemented instead with 15 g inulin per day during the last fermentation period (Inulin). Effluents (13 ml) were simultaneously sampled daily in the morning from each reactor and processed within 1 h for the enumeration of *S. Typhimurium* N-15 by selective plating and of bacterial populations by real-time quantitative PCR (real-time qPCR) analysis, and for HPLC measurements.

Salmonella enumeration by plate counts. Serial 10-fold dilutions of effluent samples were carried out in peptone water (0.1 %, pH 7.0) and plated daily in duplicate on CHROMagar *Salmonella* medium (Becton Dickinson). Plates were incubated aerobically at 37 °C for 48 h.

Fermentation 1 (F1)

Fermentation 2 (F2)

Fig. 1. Experimental design for continuous intestinal fermentations. Stab, stabilization; Sal, *S. Typhimurium* N-15; Ecol I and Ecol II, *E. coli* L1000 *wt*; Ecol I and Ecol II, *E. coli* L1000 *MccB17*–; Bif, Bif I and Bif II, *B. thermophilum* RBL67; Inulin, inulin period. †Addition of *S. Typhimurium* N-15 beads; §addition of *E. coli* L1000 beads; ‡addition of *B. thermophilum* RBL67 beads.

Analysis of gut microbiota populations using real-time qPCR.

Genomic DNA was extracted from 1.5 ml effluent samples with the FastDNA SPIN Kit for Soil (MP Biomedicals) according to the manufacturer's instructions, with a final elution volume of 100 μ l. Primer sets used for different bacterial groups in this study (Microsynth) are listed in Table 1. Primers used for the enumeration of *E. coli* L1000 *wt* and *E. coli* L1000 *MccB17*[−] were designed using the Primer3 Input software (version 0.4.0; <http://frodo.wi.mit.edu/primer3>) based on the *mcbABCDEFGF* operon sequence (EMBL accession no. FM877811) and the sequence in the mutant strain after removal of the selection marker. Primers were checked for T_m (melting temperature), secondary structure formation, G + C content and primer–dimer formation using the PCR Primer Stats software (http://www.bioinformatics.org/sms2/pcr_primer_stats.html).

PCR amplification and detection were performed with an ABI PRISM 7500-PCR sequence detection system (Applied Biosystems) in MicroAmp Fast Optical 96-Well Reaction Plates with Barcode (Applied Biosystems). Each reaction was run in duplicate and took place in a total volume of 25 μ l, containing 12.5 μ l 2 $\times$ SYBR Green PCR Master Mix (Applied Biosystems), 0.2 μ mol l^{−1} of the appropriate primers, and 1 μ l template DNA diluted 10- or 100-fold depending on the targeted bacterial group. For the detection of *B. thermophilum* RBL67, reaction mixtures were composed of 12.5 μ l qPCR MasterMix Plus LowRox (Eurogentec), 2.5 μ l template DNA diluted 10-fold, 0.3 μ mol l^{−1} of each primer and 0.1 μ mol l^{−1} TaqMan probe. Real-time qPCR conditions were kept at the pre-settings of the ABI PRISM 7500-PCR sequence detection system, with an initial heating step of 2 min at 50 °C and a denaturation step of 10 min at 95 °C, followed by 40 amplification cycles of 95 °C for 15 s and 60 °C for 1 min. A final dissociation step was added for SYBR Green amplifications to improve amplification specificity. The fluorescent products were detected during the second step of each cycle. Melting curve analysis was performed after amplification using the 7500 Fast system SDS Software (version 1.4, Applied Biosystems) to evaluate the specificity of the primers and quality of the PCR. A standard curve for each primer pair was analysed in the same run and used for calculation of the number of 16S rDNA or gene copies detected in each sample. Mean copy number per millilitre effluent (MCN ml^{−1}) was calculated for the last 3 days of each period.

Metabolite analysis. Short-chain fatty acid (SCFA; acetate, propionate and butyrate) concentrations in effluent samples were determined by HPLC analysis, as described previously (Cleusix *et al.*, 2008).

Statistical analysis. All statistical analyses were performed using the statistical software JMP 8.0 for Windows (SAS Institute). Cell counts and MCN ml^{−1} values were log₁₀-transformed to stabilize the variance and normalize the residual values for variance homogeneity.

The significance of slopes and intercepts of regression curves of log-transformed *Salmonella* cell concentrations in effluent samples versus time were tested for their difference from 0 using the *t* test ($P < 0.05$). A multivariate analysis of variance (MANOVA) was performed to compare slopes of trend lines between reactors using the *F* test ($P < 0.05$).

A one-way analysis of variance (ANOVA) was performed to test differences between mean metabolite concentrations measured by HPLC in effluent samples collected for the last 3 days of the experiment. An ANOVA was performed to test the effects of different treatments on mean log-transformed cell counts of *Salmonella* measured by selective plating and mean log-transformed MCN ml^{−1} of *E. coli* L1000 *wt* or *MccB17*[−] and *B. thermophilum* RBL67 (mean of the last 3 days of the periods), and on mean log-transformed MCN ml^{−1} of intestinal bacterial groups (mean of the last 3 days and three reactors) detected by real-time qPCR analyses. Means were compared using the Tukey–Kramer HSD test with a probability level of $P < 0.05$.

RESULTS

Mutant construction

The microcin B17 activity of *E. coli* L1000 *wt* and its mutant *MccB17*[−] was tested with the double-agar layer assay (Fig. 2). *E. coli* L1000 *wt* was highly active against the indicator organism *E. coli* NCTC 50154 and also inhibited *S. Typhimurium* N-15. In contrast, no inhibition was detected for the mutant strain *E. coli* L1000 *MccB17*[−] with both indicator strains.

Colonization of *E. coli* L1000 and *B. thermophilum* RBL67

The log₁₀(MCN ml^{−1}) of intestinal populations was measured by real-time qPCR analysis and calculated for the last 3 days of each fermentation period. The MCN values for *Enterobacteriaceae* and *Bifidobacterium* spp. and subpopulations of *E. coli* L1000 and *B. thermophilum* RBL67 are shown in Figs 3 and 4, respectively.

No signal was detected for *E. coli* L1000 *wt* and *MccB17*[−] in effluent samples before addition to F1 and F2, respectively. After adding 5 g *E. coli* L1000 *wt* (F1) and *MccB17*[−] (F2) beads to R1 (Ecol I and Ecol*I), MCN values were 1–1.5 log units higher for *E. coli* L1000 *wt* compared with *MccB17*[−], which was significant only in R2 and R3 ($P < 0.05$). A second addition of *E. coli* L1000 beads to R3 in both systems led to a significant ($P = 0.011$) increase of *E. coli* L1000 counts in the effluent of R3 only for the mutant strain *MccB17*[−] (F2), with no effect for *E. coli* L1000 *wt*. Counts of *E. coli* L1000 *wt* decreased in both R1 and R2, and increased in R3 ($P < 0.05$) by approximately 1 log unit after adding *B. thermophilum* RBL67 to R1 (Bif I). In contrast, no change of *E. coli* L1000 counts was observed for the mutant strain *MccB17*[−] (Bif in

Fig. 2. Double-agar layer activity assays for *E. coli* L1000 *wt* (a, b) and *E. coli* L1000 *MccB17*[−] (c, d) against target organisms *E. coli* NCTC 50154 (a, c) and *S. Typhimurium* N-15 (b, d).

Fig. 3. Mean concentrations of *Enterobacteriaceae* (■, □), *E. coli* L1000 wt (●) and *E. coli* L1000 MccB17- (○) in effluent samples of the proximal (R1), transverse (R2) and distal (R3) colon reactors of F1 (filled symbols) and F2 (open symbols) measured by real-time qPCR, expressed as log₁₀(MCN ml⁻¹) (mean of last 3 days of the period). Period means with different letters are significantly different within F1 (roman type) and F2 (italic type), respectively, with the Tukey-Kramer HSD test ($P < 0.05$). Asterisks signify that means for the corresponding periods of F1 and F2 are significantly different with the F test.

F2) and after a second addition of *B. thermophilum* RBL67 to R1 (Bif II in F1). Inulin addition ('Inulin' in F2) induced a decrease of *E. coli* L1000 MccB17- counts in R1 and R2 (only significant for R1), with no change recorded in R3.

B. thermophilum RBL67 was not detected in effluent samples of F1 and F2 before addition to R1. After adding 5 g *B. thermophilum* RBL67 beads to R1, counts of *B.*

Fig. 4. Mean concentrations of *Bifidobacterium* spp. (■, □) and *B. thermophilum* RBL67 (●, ○) in effluent samples of the proximal (R1), transverse (R2) and distal (R3) colon reactors of F1 (filled symbols) and F2 (open symbols) measured by real-time qPCR, expressed as log₁₀(MCN ml⁻¹) (mean of last 3 days of the period). Period means with different letters are significantly different within F1 (roman type) and F2 (italic type), respectively, with the Tukey-Kramer HSD test ($P < 0.05$).

thermophilum RBL67 reached similar high values in all reactors of F1 and F2, with respective mean values for log₁₀(MCN ml⁻¹) of 6.5 ± 0.4 (Bif I) and 6.5 ± 0.1 (Bif). After a second addition of *B. thermophilum* RBL67 beads to R1 of F1 (Bif II), counts only increased significantly ($P=0.035$) in R3 compared with the previous period (Bif I), whereas no effect was detected for R1 and R2. Addition of inulin to the growth medium led to a sharp and significant ($P < 0.007$) increase of *B. thermophilum* RBL67

counts in all reactors of F2 of up to $3 \log_{10}(\text{MCN ml}^{-1})$ units.

Effects of probiotic and prebiotic treatments on *Salmonella* propagation

Daily *Salmonella* concentrations determined by selective plating during 65-day fermentations in F1 and F2 are presented in Fig. 5, and mean concentrations detected during the last 3 days of each fermentation period are displayed in Table 2.

After the first addition of 2 g *S. Typhimurium* N-15 beads, *Salmonella* was detected in the effluents of all reactors after 1 day but decreased steadily thereafter. A second addition of 2.5 g *S. Typhimurium* N-15 beads was performed and a

continuous increase of *Salmonella* counts was recorded in all reactors of F1 and F2, reaching at the end of the *Salmonella* stabilization period (Sal) higher levels in R1 and R2 compared with R3, with means of 5.8 ± 0.3 , 5.6 ± 0.5 and $4.3 \pm 0.5 \log \text{c.f.u. ml}^{-1}$, respectively.

Effects of *E. coli* L1000. The addition of 5 g *E. coli* L1000 wt or *MccB17*– beads to R1 further stimulated *Salmonella* growth during the following 14-day period in F1 and F2 (Fig. 5). *Salmonella* counts increased linearly with time ($P < 0.0001$) in all reactors, with a higher rate in R2 compared with R1 and R3 for both models. A second addition of 5 g *E. coli* L1000 beads in the distal reactor (R3) of F1 and F2 on day 36 had no significant effect on *Salmonella* counts in the reactor effluent (Ecol II and Ecol*II). After *E. coli* L1000 addition, *Salmonella* counts in

Fig. 5. *Salmonella* concentrations, as $\log_{10}[\text{c.f.u. (ml effluent)}^{-1}]$, measured daily by plate counts in effluents of the proximal (R1), transverse (R2) and distal (R3) colon reactors of fermentations F1 and F2. Regression lines were calculated over the time period from day 14 to 37 for F1 (solid lines) and F2 (dashed lines). * and §, addition of 5 g *E. coli* L1000 wt and *E. coli* L1000 *MccB17*– beads to F1 and F2, respectively; §§ addition of 5 g *B. thermophilum* RBL67 beads; † addition of 5 g *B. thermophilum* RBL67 beads to F1 and start of nutritive medium supplementation with inulin (15 g per day) for F2.

Table 2. *Salmonella* counts [$\log_{10}(\text{c.f.u. ml}^{-1})$] obtained by selective plating on CHROMagar *Salmonella* medium for different fermentation periods of continuous cultures in F1 and F2

Data are reported as mean $\pm$ SD of three reactors for the last 3 days. Values with different letters in a column are significantly different by the Tukey–Kramer HSD test or the *F* test ($P < 0.05$). ND, Not detected.

Period	R1	R2	R3
F1			
Stab	ND	ND	ND
Sal	5.6 ± 0.2^c	5.9 ± 0.2^c	4.7 ± 0.1^b
Ecol I	6.8 ± 0.4^{ab}	7.6 ± 0.1^a	6.3 ± 0.1^a
Ecol II	6.5 ± 0.2^b	7.3 ± 0.1^{ab}	6.4 ± 0.2^a
Bif I	7.2 ± 0.2^{ab}	6.8 ± 0.4^b	6.1 ± 0.6^a
Bif II	7.3 ± 0.2^a	7.1 ± 0.1^{ab}	6.3 ± 0.1^a
F2			
Stab	ND	ND	ND
Sal	6.0 ± 0.1^b	5.3 ± 0.6^b	4.0 ± 0.4^c
Ecol*I	6.6 ± 0.1^{ab}	7.1 ± 0.2^a	6.0 ± 0.3^b
Ecol*II	6.4 ± 0.2^{ab}	7.2 ± 0.1^a	6.3 ± 0.2^b
Bif	6.9 ± 0.1^a	6.8 ± 0.4^a	6.5 ± 0.3^b
Inulin	6.8 ± 0.4^a	6.9 ± 0.2^a	7.2 ± 0.2^a

all reactors of F1 and F2 were equal or higher to $6.0 \log_{10}(\text{c.f.u. ml}^{-1})$ (Table 2).

Effects of *B. thermophilum* RBL67. Addition of 5 g *B. thermophilum* RBL67 beads on day 44 to R1 showed no significant effects on *Salmonella* counts in all three reactors

of F1 and F2, which were in the range from 6.1 ± 0.6 to $7.2 \pm 0.2 \log_{10}(\text{c.f.u. ml}^{-1})$ during the last 3 days of the Bif I (F1) and Bif (F2) periods (Table 2). Subsequent addition of 5 g *B. thermophilum* RBL67 beads to R1 on day 55 had no significant effect on *Salmonella* counts (Bif II, F1) compared with the previous Bif I period.

Effects of inulin. Supplementation of the nutritive medium with inulin during the final experimental period of F2 (Inulin) led to significantly ($P = 0.037$) increased levels of *Salmonella* in R3, with no detectable effects in R1 and R2.

Effects of *Salmonella*, probiotic and prebiotic treatments on main gut microbiota populations

MCN (ml effluent^{-1}) values of intestinal populations measured by real-time qPCR during the last 3 days of each experimental period were not significantly different for the proximal, transverse and distal reactors. Therefore, the mean MCN values calculated for the three reactors are presented in Table 3.

Independent of the experimental period, high and stable counts of total bacteria were detected in all reactors of both models, with high values in the range 2.6×10^{10} – 5.8×10^{10} and 1.5×10^{10} – 4.7×10^{10} MCN ml^{-1} measured for F1 and F2, respectively, during 65 days of continuous culture. *Bacteroides* spp. was the predominant bacterial group, followed by *Enterobacteriaceae*, *Bifidobacterium* spp. and *Lactobacillus/Leuconostoc/Pediococcus* spp. Counts of *Bacteroides* spp. were stable overall during the experiment in F1 and F2, except for the final inulin period in R1 of F2,

Table 3. $\log_{10}(\text{MCN ml}^{-1})$ values for intestinal populations measured by real-time qPCR in faecal inoculum and fermentation effluent samples for different periods of fermentations F1 and F2

Data are means $\pm$ SD of three reactors calculated for the last 3 days of each fermentation period. Values with different letters in a column of the same reactor are significantly different by the Tukey–Kramer HSD test ($P < 0.05$).

Period	Total bacteria	<i>Bacteroides</i> spp.	<i>Bifidobacterium</i> spp.	<i>Lactobacillus/Leuconostoc/Pediococcus</i> spp.	<i>Enterobacteriaceae</i>
Faeces	10.7	10.1	7.6	5.1	5.5
F1					
Stab	10.4 ± 0.1^a	9.5 ± 0.3^a	7.2 ± 0.3^a	3.4 ± 0.3^d	8.3 ± 0.3^a
Sal	10.5 ± 0.2^a	9.6 ± 0.4^a	7.2 ± 0.3^a	4.4 ± 0.5^c	8.6 ± 0.3^a
Ecol I	10.5 ± 0.2^a	9.6 ± 0.3^a	7.0 ± 0.3^{ab}	5.4 ± 0.6^b	8.5 ± 0.2^a
Ecol II	10.5 ± 0.1^a	9.6 ± 0.3^a	6.7 ± 0.2^{bc}	6.0 ± 0.3^b	8.5 ± 0.3^a
Bif I	10.6 ± 0.2^a	9.7 ± 0.2^a	6.8 ± 0.3^{ab}	5.7 ± 0.3^b	8.5 ± 0.5^a
Bif II	10.5 ± 0.2^a	9.6 ± 0.3^a	6.4 ± 0.2^c	6.7 ± 0.2^a	8.3 ± 0.2^a
F2					
Stab	10.4 ± 0.2^a	9.4 ± 0.5^a	6.9 ± 0.5^b	3.2 ± 0.3^c	8.3 ± 0.4^{bc}
Sal	10.5 ± 0.2^a	9.4 ± 0.4^a	6.6 ± 0.4^b	4.3 ± 0.6^d	8.7 ± 0.2^{ab}
Ecol*I	10.4 ± 0.2^a	9.4 ± 0.4^a	6.5 ± 0.2^{bc}	5.1 ± 0.5^c	8.3 ± 0.3^{bc}
Ecol*II	10.4 ± 0.2^a	9.5 ± 0.4^a	6.1 ± 0.2^c	5.1 ± 0.8^c	8.7 ± 0.2^a
Bif	10.4 ± 0.2^a	9.6 ± 0.3^a	6.5 ± 0.4^{bc}	5.9 ± 0.2^b	8.4 ± 0.2^{abc}
Inulin	10.4 ± 0.2^a	$9.1 \pm 0.9^{a*}$	8.3 ± 0.2^a	8.1 ± 0.3^a	8.1 ± 0.3^c

* $\log_{10}(\text{MCN ml}^{-1})$ values were significantly lower in R1 (8.0 ± 0.4) than in R2 (9.7 ± 0.3) and R3 (9.8 ± 0.1).

Table 4. Mean SCFA concentrations (mmol l⁻¹) and SCFA ratios in effluents sampled from the proximal (R1), transverse (R2) and distal (R3) colon reactors of fermentations F1 and F2

Data are means $\pm$ SD and ratios (in parentheses) calculated for the last 3 days of each fermentation period and for the overall experiment. Values with different letters in a column of the same reactor are significantly different by the Tukey–Kramer HSD test ($P < 0.05$).

Reactor	Total SCFA	Acetate	Butyrate	Propionate
F1				
R1	140.6 $\pm$ 5.5 ^c	76.7 $\pm$ 4.7 ^c (55)	46.2 $\pm$ 3.8 ^c (33)	17.7 $\pm$ 2.5 ^c (13)
R2	171.2 $\pm$ 5.3 ^b	93.3 $\pm$ 4.6 ^b (54)	53.1 $\pm$ 3.7 ^b (31)	24.8 $\pm$ 3.3 ^b (14)
R3	190.1 $\pm$ 8.5 ^a	104.4 $\pm$ 5.7 ^a (55)	56.7 $\pm$ 4.7 ^a (30)	29.0 $\pm$ 2.4 ^a (15)
F2				
R1	142.6 $\pm$ 17.6 ^b	74.0 $\pm$ 6.1 ^b (52)	55.8 $\pm$ 15.9 ^a (39)	12.8 $\pm$ 2.0 ^c (9)
R2	184.7 $\pm$ 28.2 ^a	94.2 $\pm$ 7.8 ^a (53)	62.0 $\pm$ 18.5 ^a (35)	22.4 $\pm$ 3.0 ^b (13)
R3	178.5 $\pm$ 26.3 ^a	94.1 $\pm$ 7.9 ^a (51)	64.7 $\pm$ 19.4 ^a (35)	25.8 $\pm$ 2.5 ^a (14)
Faeces	125.0	80.4 (64)	21.2 (17)	23.3 (19)

where a significant ($P = 0.001$) decrease from 9.4 ± 0.4 to 7.9 ± 0.4 log₁₀(MCN ml⁻¹) was measured. MCN ranges of bacterial groups measured during Stab in the effluents of the different reactors were similar to those of the faecal inoculum, except for *Enterobacteriaceae*, for which the MCN values were about 3 log units higher than for the faecal inoculum.

Salmonella addition to F1 and F2 had no significant ($P > 0.05$) effects on *Bifidobacterium* spp. and *Enterobacteriaceae* populations, but was accompanied by a significant increase of *Lactobacillus/Leuconostoc/Pediococcus* spp. counts, from 3.4 ± 0.3 (Stab) to 4.4 ± 0.5 (Sal) log₁₀(MCN ml⁻¹) in F1 ($P = 0.003$) and from 3.2 ± 0.3 (Stab) to 4.3 ± 0.6 (Sal) log₁₀(MCN ml⁻¹) in F2 ($P = 0.012$).

Effects of *E. coli* L1000. No significant ($P > 0.05$) changes of *Bifidobacterium* spp. and *Enterobacteriaceae* populations were measured during the first *E. coli* L1000 treatments [Ecol I (F1) and Ecol* I (F2)], and MCN values of *E. coli* L1000 were 3–5 log units lower compared with total *Enterobacteriaceae*. MCN values for *Lactobacillus/Leuconostoc/Pediococcus* spp. increased significantly ($P < 0.0001$) with the first addition of *E. coli* L1000 wt (F1, +1 log unit) or *MccB17*– (F2, +0.8 log unit) to levels similar ($P > 0.05$) to those measured in the faecal inoculum [5.1 log₁₀(MCN ml⁻¹)]. No significant population changes were detected after the second addition of *E. coli* L1000, except for a small but significant ($P = 0.032$) increase of *Enterobacteriaceae* from 8.3 ± 0.3 (Ecol*I) to 8.7 ± 0.2 log₁₀(MCN ml⁻¹) (Ecol*II) in F2.

Effects of *B. thermophilum* RBL67. The first addition of *B. thermophilum* RBL67 to F1 (Bif I) and F2 (Bif) had no significant ($P > 0.05$) effects on *Bifidobacterium* spp. and *Enterobacteriaceae* populations. Counts of *B. thermophilum* RBL67 during these periods were equal to total *Bifidobacterium* spp. counts. *Lactobacillus/Leuconostoc/Pediococcus* counts increased significantly ($P = 0.032$) only in F2 compared with the previous Ecol*II period, and stabilized at 5.8 ± 0.3 log₁₀(MCN ml⁻¹) (Bif I, Bif) in both F1 and F2. In

contrast, the second addition of *B. thermophilum* RBL67 in F1 (Bif II) was accompanied by a small ($P = 0.029$) decrease of *Bifidobacterium* spp. from 6.8 ± 0.3 (Bif I) to 6.4 ± 0.2 (Bif II) log₁₀(MCN ml⁻¹) and a significant ($P < 0.0001$) increase of *Lactobacillus/Leuconostoc/Pediococcus* spp. from 5.7 ± 0.3 (Bif I) to 6.7 ± 0.2 (Bif II) log₁₀(MCN ml⁻¹).

Effects of inulin. Inulin addition in F2 resulted in a sharp and significant ($P < 0.0001$) increase of both *Lactobacillus/Leuconostoc/Pediococcus* spp. and *Bifidobacterium* spp. populations [>2 and 1.5 log₁₀(MCN ml⁻¹), respectively, in all reactors], concomitant with *B. thermophilum* RBL67 counts.

Effects of probiotic and prebiotic treatments on gut metabolic activity

No periodic effects (except for inulin addition) were detected on SCFA concentrations and ratios measured by HPLC. Therefore, mean SCFA concentrations and ratios calculated for the last 3 days of all experimental periods for different reactors are presented in Table 4. Stable levels of total SCFA concentrations were measured in all reactors for both F1 and F2, with increasing values from the proximal (R1) to the distal (R3) colonic reactors. SCFA concentrations and ratios were similar for F1 and F2 (with the exception of the last inulin period; Table 4). Inulin addition in F2 led to a significant ($P < 0.05$) increase in butyrate from 46.8 ± 0.9 , 54.3 ± 1.5 and 52.4 ± 0.5 mmol l⁻¹ to 88.4 ± 1.8 , 105.1 ± 6.0 and 101.1 ± 3.8 mmol l⁻¹ in R1, R2 and R3, respectively. Butyrate accounted for approximately 50 % of total SCFA compared with only 35 % during the previous Bif period. A concomitant reduction in proportion of acetate from 56 to 44 % was measured, whereas propionate remained unchanged.

DISCUSSION

The development of biological alternatives to antibiotics in order to treat infections and address the increasing

prevalence of antibiotic-resistant and highly invasive pathogenic bacteria is of high public interest. Probiotic bacteria have great potential to complement or substitute conventional antibiotic treatments. Beneficial properties such as anti-*Salmonella* activity are, however, strain-specific and must be assessed separately for each probiotic bacterium. Two promising probiotic strains isolated from human faeces were tested in this study, *E. coli* L1000 and *B. thermophilum* RBL67 (von Ah *et al.*, 2007; Zihler *et al.*, 2009). These have been shown to produce proteinaceous antimicrobial compounds with high and broad activity against *Salmonella* when assessed with simple antagonistic tests.

The human colon constitutes a protective and nutrient-rich habitat for trillions of bacteria living in symbiosis with the host (Gaskins *et al.*, 2008). This dense and host-dependent microbial community has established an ecological mutualism with the host that confers a powerful barrier effect against pathogenic invaders, referred to as colonization resistance (Barth *et al.*, 2009). The ability to protect the host from invading pathogens presupposes that probiotics are able to transiently occupy a specific ecological niche in adequate numbers for optimal functionality. Barth *et al.* (2009) were able to show recently that the probiotic *E. coli* strain Nissle 1917 producing colicin has the capacity to adapt easily to the intestinal milieu of healthy conventional piglets, allowing persistence and replication in the gut. There are, however, no studies investigating the competitiveness of bacteriocinogenic strains compared with negative mutants for intestinal colonization and efficacy to counteract pathogens. In our study, *E. coli* L1000, independently of the presence of genetic determinants for microcin B17 production, was not able to colonize *in vitro* a complex gut microbiota at a high level [$\leq 5.8 \log_{10}(\text{MCN ml}^{-1})$ during the Ecol periods], even after high inoculation with 6×10^9 cells immobilized in beads to induce colonization. This addition considerably exceeds the colonic delivery of probiotics at the recommended daily dose of 10^8 – 10^9 cells believed to exert a beneficial effect on the host (Gill & Prasad, 2008), while accounting for the potentially high viability loss during passage through the upper gastrointestinal tract. Accordingly, *E. coli* L1000 addition did not significantly affect gut microbiota composition or metabolic activity, except for an increase of the *Lactobacillus/Leuconostoc/Pediococcus* population, although it strongly stimulated *Salmonella* growth. It has been previously shown that addition of *Salmonella* to a proximal reactor of an *in vitro* intestinal colonic fermentation model with immobilized child faecal microbiota stimulates the growth of the *Clostridium coccoides*–*Eubacterium rectale* group (Le Blay *et al.*, 2009), which also belongs to the phylum Firmicutes. The inability of *E. coli* L1000 to stabilize at high levels in our model could also be explained by the high levels of *Enterobacteriaceae* with similar environmental requirements in the intestinal ecosystem of the donor. Microcin B17 production did not confer a significant ecological

advantage upon *E. coli* L1000 wt over other *Enterobacteriaceae*, although microcin B17 has been shown to be active against *Salmonella* and other *Enterobacteriaceae* (Pons *et al.*, 2002). However, the *E. coli* L1000 wt population was significantly higher in R2 and R3 after the first inoculation of the strain in R1 (Ecol I) compared with the bacteriocin-negative mutant (Ecol*I), possibly due to *in situ* microcin B17 production. Stecher *et al.* (2010) have recently provided evidence that the presence of closely related strains can enhance the possibility of invasion of exogenous bacteria into the gut ecosystem. Mice harbouring high levels of gut lactobacilli are more efficiently colonized by a commensal and orally administered *Lactobacillus reuteri*^{RR} strain. In addition, those authors provide evidence that individuals with high commensal *Enterobacteriaceae* densities are more susceptible to *Salmonella*-induced gut inflammation. Our data that show a stimulation of *Salmonella* growth after addition of *E. coli* L1000 to a gut microbiota already highly colonized with *Enterobacteriaceae* support this mechanism.

In contrast to *E. coli* L1000, *B. thermophilum* RBL67 colonized the intestinal microbiota at high levels after the first addition and accounted for a very high proportion (~70 %) of the total bifidobacteria population. In a previous study, we showed that *B. thermophilum* RBL67 added to a reactor simulating a child proximal colon made up to 12 % of total bifidobacteria (A. Zihler and others, unpublished results). Although the metabolic activity and composition of the intestinal ecosystem were little changed upon *B. thermophilum* RBL67 addition, a significant decrease in bifidobacteria counts was observed, possibly explained by thermophilicin B67 production affecting closely related bacteria. In addition, *B. thermophilum* RBL67 was highly active against *S. Typhimurium* M557, when added to the reactor preventively before or curatively after *Salmonella* colonization. Compared with the present study, *Salmonella* counts were about 1.5 log units lower before the addition of strain RBL67. Therefore, very different *B. thermophilum* RBL67: *Salmonella* ratios (3050:1 and 2:1, respectively) were reached. The determination of the required probiotic dose for a targeted anti-pathogenic effect is difficult because the optimal concentration depends on several factors, including the probiotic organism, duration of application, delivery matrix (food or pharmaceutical) and the targeted population group (de Vrese & Schrezenmeir, 2008). For example, a dose-efficacy study revealed that a 1-week *Lactobacillus rhamnosus* GG (LGG) treatment was equally effective at two dosages (10^{10} and 10^{12} c.f.u. twice per day) in decreasing the frequency and duration of rotavirus-induced acute watery diarrhoea in Indian children (Basu *et al.*, 2009), although data on the LGG:rotavirus ratios in the gut are lacking. To our knowledge, no study has investigated probiotic: pathogen ratios in the gut for maximal enteric protection, especially against *Salmonella*.

Inulin addition produced a large increase of lactobacilli counts and a pronounced bifidogenic effect accompanied by a shift in SCFA ratios towards elevated butyrate levels, as observed in previous studies (Kleessen *et al.*, 2001;

Langlands *et al.*, 2004; Le Blay *et al.*, 2009). Interestingly, addition of inulin also led to a sharp increase of *B. thermophilum* RBL67 counts in all three reactors, and stimulated the growth of *Salmonella* in the distal colon reactor R3. Several studies have reported a correlation between the administration of different prebiotics (including inulin) and an enhanced severity of *Salmonella* infection in rats and mice (Bovee-Oudenhoven *et al.*, 2003; Petersen *et al.*, 2009; Ten Bruggencate *et al.*, 2003, 2004) with increased translocation of *Salmonella*, although these effects are not always correlated with an enhanced pathogen colonization of the gut.

In conclusion, data obtained with our *in vitro* intestinal fermentation model of *Salmonella* colonization in the child colon reflect a lack of efficacy of microcin B17-producing *E. coli* L1000 to inhibit *Salmonella*, in contrast to the high efficacy displayed by this strain in simple *in vitro* antagonistic tests (Zihler *et al.*, 2009). Although simple *in vitro* activity assays are practical and useful for first screening of probiotics active against *Salmonella*, more complete models reflecting the complex gut microbiota and environments are required to assess their efficacy and provide information on the mechanisms of probiotics for enteric protection within a complex microbial ecosystem. This validation step is important before considering *in vivo* testing with animal models. Indeed, several biotic factors are difficult to reproduce *in vitro*, and especially host immune and neuroendocrine responses are lacking in *in vitro* colonic fermentation models. Therefore, we are currently testing with intestinal epithelial cell models the *in vitro* effects on the host cell response of effluent samples produced by the colonic fermentation model and containing a child gut microbiota, *Salmonella* and probiotics. Such combined *in vitro* models could provide a more complete assessment of the potential and mechanisms of probiotics against pathogen infection in the gut.

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