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New *in vitro* colonic fermentation model for *Salmonella* infection in children gut

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Abstract

In this study, a new *in vitro* continuous colonic fermentation model of *Salmonella* infection with immobilized child fecal microbiota and *Salmonella* serovar Typhimurium was developed for the proximal colon. This model was then used to test the effects of two amoxicillin concentrations (90 and 180 mg day^{-1}) on microbial composition and metabolism of the gut microbiota and on *Salmonella* serovar Typhimurium during a 43-day fermentation. Addition of gel beads (2%, v/v) colonized with *Salmonella* serovar Typhimurium in the reactor resulted in a high and stable *Salmonella* concentration (log 7.5 cell number mL^{-1}) in effluent samples and a concomitant increase of *Enterobacteriaeceae*, *Clostridium coccoides- Eubacterium rectale* and *Atopobium* populations and a decrease of bifidobacteria. During amoxicillin treatments, *Salmonella* concentrations decreased while microbial balance and activity were modified in agreement with *in vivo* data with a marked decrease of *Clostridium coccoides-Eubacterium rectale* and an increase in *Enterobacteriaceae*. After interruption of antibiotic addition, *Salmonella* concentration increased again to reach values comparable to that measured before antibiotic treatments, showing that our model can be used to simulate *Salmonella* shedding in children as observed *in vivo*. This *in vitro* model could be a useful tool for developing and testing new antimicrobials against enteropathogens.

Introduction

Salmonellosis is one of the most common and widely distributed foodborne diseases worldwide. It is associated with two types of symptoms, caused by different serovars of *Salmonella enterica* subsp. *enterica*: typhoid fever (mainly caused by serovar Typhi, Paratyphi and Sendai) which is more common in developing countries; and gastroenteritis (mainly caused by serovar Typhimurium and Enteritidis) also encountered in developed countries (Coburn *et al.*, 2007). In Europe, 176,395 cases of salmonellosis infections (i.e. 38 for 100 000 habitants) have been declared in 2005, with a major proportion (20%) of young children less than 5 years of age (The European Food Safety Authority & European Center for Disease Prevention and Control, 2006). In children, dehydration associated with diarrhea can become severe and life-threatening (Rosanova *et al.*, 2002), therefore effective antimicrobials are essential drugs for treatment. Antimicrobials most widely regarded as optimal for treating diarrheal diseases caused by *Salmonella* serovar Typhimurium in children are third-generation cephalosporins, because quinolones and fluoroquinolones are generally not recommended due to their toxicity on immature cartilage and the possible emergence of resistant pathogens (Schaad, 2005). The earlier drugs, chloramphenicol, amipicillin, amoxicillin and trimethoprim-sulfamethaoxazole, are also used as alternatives (Frye & Fedorka-Cray, 2007; World Health Organization, 2008). However, the emergence of *Salmonella* isolates with multiple-drug resistance urges the need for alternatives to antibiotherapies (White *et al.*, 2001; World Health Organization, 2008). Valuable models of *Salmonella* infection are therefore needed to develop and test new treatments, in particular for young human populations.

To our knowledge, there is currently no suitable model to test the effects of new antimicrobials on both enteropathogens and intestinal bacteria. Animal models, such as bovine or streptomycin-pretreated mice models for *Salmonella* infection do not reproduce the human intestinal microbiota and are dedicated for studying host-pathogen interactions

(Hapfelmeier & Hardt, 2005). Moreover, *in vivo* studies are difficult to perform due to cost, ethical problems and high interindividual variations. By contrast, *in vitro* models are much less expensive, simpler to handle and could be a good alternative for testing new antimicrobial treatments in a first screening phase; but current models are not suitable for intestinal infections. The continuous culture models for human intestinal microbiota are mainly based on the original model of Macfarlane *et al.* (1998) and inoculated with diluted feces. These systems have several limitations due to the planktonic state of bacterial populations, leading to limited microbial stability and cell density compared to the colon; they are also not suited for long-term experiments with enteropathogens since exogenous bacteria are rapidly washed out of the system (Blake *et al.*, 2003; Carman *et al.*, 2004; Carman & Woodburn, 2001; Payne *et al.*, 2003). Recently, we developed and validated a new model of infant and adult colonic fermentation with fecal microbiota immobilized in gel beads in anaerobic continuous-flow cultures (Cinquin *et al.*, 2006a; Cinquin *et al.*, 2006b; Cleusix *et al.*, 2008; Macfarlane & Macfarlane, 2007). This new model has conditions that are more akin to that of the intestinal system and has the following characteristics: bacteria growing in biofilm structures; high cell density in gel beads and in reactor effluents (up to 10^{11} cells mL^{-1} or g^{-1}); high stability and reactivity to changing conditions of the intestinal fermentation; good protection of sensitive bacteria from shear and oxygen stresses; and prevention of washout and loss of less competitive bacteria.

In this study we developed an *in vitro* model of intestinal fermentation with immobilized feces simulating intestinal *Salmonella* infections and long-term shedding in children. We hypothesized that addition of colonized beads with *Salmonella* serovar Typhimurium in the intestinal fermentation model containing an immobilized child microbiota would cause a stable infection of *Salmonella* in gel beads and in effluent samples. The effect of two antibiotic treatments on *Salmonella* serovar Typhimurium, as well as on the main bacterial

populations and metabolism of child's microbiota were tested during the same continuous culture and compared with *in vivo* data to validate the model.

Materials and methods

Bacterial strain

Salmonella enterica subsp. *enterica* serovar Typhimurium M557 *(sseD::aphT ⊿invG)* (*S.* Typhimurium) was supplied by Prof. W. Hardt (Institute of Microbiology, ETH, Zurich, Switzerland). Sensitive to amoxicillin, this strain is a low virulent derivative of *S.* Typhimurium wild-type strain SL1344 lacking SPI-1 effector proteins (Hapfelmeier *et al.*, 2004). It was routinely cultivated in tryptone soya broth (TSB, Oxoid, Basel, Switzerland) overnight at 37 °C in aerobiosis.

Antibiotic

In human practice, Clamoxyl®/-RC (GlaxoSmithKline) containing amoxicillin as active compound, is a moderate-spectrum β-lactam antibiotic that can be used to treat salmonellosis in children (25-75 mg kg^{-1} day^{-1}) (Moulin *et al.*, 2003; Fachinformation des Arzneimittel-Kompendium der Schweiz®, 2007). It is bactericidal against a wide range of Gram-positive and Gram-negative bacteria, including sensitive *Salmonella* with minimum inhibitory concentrations (MIC) ranging from 1 to 5 µg mL^{-1} for 90% of tested strains (Fachinformation des Arzneimittel-Kompendium der Schweiz®, 2007). For the experiment, pure amoxicillin (Sigma-Aldrich, Buchs, Switzerland) was used.

Feces collection and bacterial immobilization

The fecal sample used for immobilization was collected from a healthy 2 year-old child, who had not received antibiotics at least 3 months before the experiment. The fecal sample was maintained in anaerobiosis and immobilized in 1-2 mm gel beads composed of gellan (2.5%,

w/v) and xanthan (0.25%, w/v) gums and sodium citrate (0.2%, w/v), as already described (Cleusix *et al.*, 2008). Gel beads (60 mL) were then transferred into a stirred glass reactor (Sixfors, Infors, Bottmingen, Switzerland) with 140 mL of fresh nutritive medium simulating a child chyme (presented below). The entire process was completed under anaerobic conditions within 3 h after defecation.

S. Typhimurium immobilization was done one day before reactor inoculation using the same procedure as for fecal samples, but in aerobiosis under a sterile bench. *Salmonella* beads (10 mL) were colonized overnight in 200 mL TSB at 37 °C in aerobiosis without pH-control. A fresh bead sample (0.5 g) was used to inoculate the reactor on day 11 and the rest was stored frozen (0.5 g aliquots) in 20% glycerol at -80 °C.

Nutritive medium

The nutritive medium used to feed the reactor was similar to that previously described by Macfarlane *et al.* (1998) for simulating an adult ileal chyme, with one modification; the bile salt concentration was reduced from 0.4 to 0.05 g L^{-1} to reproduce the ileal chyme of a young child. A solution of vitamins described by Michel *et al.* (1998) and sterilized by filtration (Minisart 0.2 µm, Sartorius, Göttingen, Germany) was added (0.5 mL l^{-1}) separately to the autoclaved (15 min, 121 °C) medium.

Experimental setup and sampling

A single-stage reactor based on the model described by Cinquin *et al.* (2004) was used to mimic the microbial ecosystem of a child's proximal colon. Batch fermentations were first carried out to colonize the fecal beads for 2 days. During colonization, the nutritive medium was aseptically replaced by fresh nutritive medium every 12 h. Temperature (37 °C) was automatically controlled and pH was maintained at 5.7 by adding NaOH (5N). Anaerobic conditions were maintained during the whole fermentation by a continuous flow of pure CO_2 in the headspace. The continuous fermentation was carried out in the same reactor connected

to a stirred feedstock vessel containing sterile nutritive medium continuously flushed with CO_2 and maintained at 4 °C and to an effluent receiving vessel. Continuous medium feeding was carried out using peristaltic pumps (Reglo analog, Ismatec, Glattbrugg, Switzerland) delivering a feed flow rate of 40 mL h^{-1} for a mean retention time of 5 h. This time was used to simulate the residence time in a child proximal colon, with a pH of 5.7 (Fallingborg *et al*, 1990).

The 43-day continuous fermentation was divided into 6 periods of 5 to 9 days (Figure 1). First the system was stabilized (STyphi 0; days 3-10), then 0.5 g of beads colonized with *S.* Typhimurium (days 11 and 13) were added to the system followed by a second stabilization period (STyphi I; days 14-20) and two antibiotic treatments (ATB I [214 µg mL^{-1} thrice a day]; days 21-25 and ATB II [428 µg mL^{-1} thrice a day]; days 35-39) intercalated with a third stabilization period without antibiotic (STyphi II, day 26-34). Amoxicillin was added directly in the reactor three times per day (at 9 a.m., 2 p.m. and 6 p.m.) to reach total concentrations of 90 mg day^{-1} (ATB I) and 180 mg day^{-1} (ATB II). According to the *Fachinformation des Arzneimittel-Kompendium Schweiz*®, the average oral dose (divided into three doses) of Clamoxyl®/- RC is 50 mg kg^{-1} day^{-1} for young children (2 to 12 year-old), with an absorption rate between 70 and 90% in the gastrointestinal tract. On the basis of two absorption rates of 70 and 85% we calculated that 90 to 180 mg day^{-1} amoxicillin should reach the colon for a 12 kg-child receiving 600 mg amoxicillin per day. On the last evening of ATB I (day 25) and II (day 39), the reactor was briefly stopped and the medium was entirely pumped out of the reactor after beads had settled. A new medium without antibiotic (Stab II and III) was immediately added. Effluent samples (10 mL) were collected daily for metabolites (short-chain fatty acids [SCFA], and lactate) and fluorescent *in situ* hybridization (FISH) analyses. The pseudo-steady state for each period was reached when bacterial populations in the reactor

effluent did not change by more than 0.5 log units during 4 consecutive days (Cinquin et al., 2006).

During antibiotic treatments, effluent samples were collected before adding the first daily antibiotic dose.

Bacterial enumeration with FISH and microscopy

FISH analyses coupled with microscopy were performed for total bacteria and *Salmonella* enumeration as described by Cinquin *et al.* (2006a) on fermentation samples (1.5 mL) from the last 3 days of each pseudo-steady-state period. Total bacteria were stained with 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, Buchs, Switzerland) and *S.* Typhimurium was targeted with Sal 3, a Cy3-labelled oligonucleotide probe (Microsynth, Balgach, Switzerland) with hybridization conditions described by Nordentoft *et al.* (1997).

Bacterial enumeration with FISH and flow cytometry

FISH analyses coupled with flow cytometry were performed based on the method described by Zoetendal *et al.* (2002) with some modifications. Briefly, 100 µL of fixed fermentation samples (1.5 mL) and fixed feces (1.5 mL) were centrifuged (9000 g, 3 min) and the pellet was washed once in Tris-EDTA buffer (100 mM Tris/HCl, 500 mM EDTA, pH 8) before incubation for 10 min at room temperature in 100 µL of Tris-EDTA buffer supplemented with lysozyme (170'800 U mL^{-1}) and proteinase K (6 µg mL^{-1}) to destroy protein-clusters formed during both antibiotic treatment periods which interfered with flow cytometry detection. After removing the lysozyme solution by centrifugation (9000 g, 3 min) and washing the pellet once with 100 µL of fresh hybridization buffer (900 mM NaCl, 20 mM Tris/HCl, 0.1% SDS, 30% formamide, pH 8), cells were resuspended in 300 µL of hybridization buffer, homogenized and divided into 10 aliquots of 25 µL. With exception of the negative control, aliquots were hybridized overnight at 35 °C with 50 ng μL^{-1} of Cy5-labeled probes (Table 1). Since hybridization conditions described for Sal 3 (Nordentoft *et al.*, 1997) slightly differed

from conditions used in flow cytometry, the Sal 3 probe specificity was tested again with hybridization conditions used for flow cytometry (Table 1).

After hybridization and to remove non-specific binding of probes, 900 µL of warm washing buffer (64 mM NaCl, 20 mM Tris/HCl, 5 mM EDTA, 0.1% SDS, pH 8) was added and samples were incubated at 37 °C for 20 min. A last centrifugation step was performed (9000 g, 3 min) before resuspending the pellet in 300 µL of cold potassium citrate buffer (10 mM Tris/HCl, 1 mM EDTA, 30 mM potassium citrate, pH 7.4). A 50 µL aliquote was diluted with 400 µL of potassium citrate buffer to obtain a final bacterial concentration of about 10^8 bacteria per mL. A volume of 0.5 µL of diluted (1/10'000) SYBR Green I (Invitrogen AG, Basel, CH) was added at least 15 min before each measurement in order to differentiate bacteria from non-bacterial material. To determine bacterial cell numbers, 50 µL of Flow-Count™ Fluorospheres (Beckman Coulter International SA, Nyon, CH) at known concentrations (1012 beads μL^{-1}) were added just before data acquisition. Samples were passed through a Cytomics FC 500 (Beckman Coulter International SA, Nyon, Switzerland) equipped with an air-cooled argon ion laser emitting 20 mW at 488 nm and a Red Solid State Diode laser emitting 25 mW at 633 nm. The 633 nm laser was used to detect red fluorescence of bacteria hybridized with Cy5-labeled probes (PMT4 in a 655 nm long pass filter) and the 488 nm laser was used to measure the forward angle light scatter (FSC), the side angle light scatter and the green fluorescence conferred by SYBR Green I (PMT1 in a 525 nm band pass filter). The acquisition threshold was set in the forward scatter channel to the minimum. The flow rate was set at 1,000-3,000 events s^{-1} and 100,000 events were stored in list mode files. Data were analyzed using the CXP software (Beckman Coulter International SA, Nyon, Switzerland). A PMT1 histogram (green fluorescence) was used to evaluate the total number of bacteria stained with SYBR Green I. In this histogram, a gate which included the total number of bacterial cells in the sample was designed and used to make a PMT4 histogram

(red fluorescence). This PMT4 histogram was then used to determine the bacterial groups marked with Cy5-labelled probes. To quantify bacterial groups and total cells, a correction was made to eliminate background fluorescence, measured using the negative control NON-EUB338-Cy5 probe, as described by Rigottier-Gois *et al.* (2003). Analyses were done in duplicate.

Metabolite analyses

SCFA (acetate, propionate, butyrate and formate) and lactate concentrations were determined by HPLC as previously described (Cleusix *et al.*, 2008). Each analysis was done in duplicate. Mean metabolite concentrations were expressed in mM.

Statistical analyses

A one-way ANOVA was performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA) to test the effects of the different treatments on bacterial and metabolite concentrations measured during the pseudo-steady-state periods (mean of 3 successive days) in effluent samples. Treatment means were compared using the Tukey's test with the probability level of $P < 0.05$. Data in the text are means ± SD.

Results

Microbial populations analyzed by FISH-flow cytometry

The child fecal sample used for immobilization showed a total population of 10.2 ± 0.1 $\log_{10}$ cells g^{-1}, and was highly dominated by bifidobacteria (9.8 ± 0.1 $\log_{10}$ cells g^{-1}). Mean concentrations of major bacterial populations measured in protease treated fermentation samples by FISH-Flow cytometry during the last three days of each experimental period are shown in Table 2. The predominant bacterial genus in the reactor effluents during the whole fermentation (except during STyphi I) was *Bifidobacterium* spp., initially followed by

Clostridium coccoides-Eubacterium rectale and *Bacteroides-Prevotella* groups. No *Salmonella* or other *Enterobacteriaceae* were detected during STyphi 0. Following addition of *S.* Typhimurium-colonized beads (11.1 $\log_{10}$ cells (g bead)$^{-1}$) to the reactor (days 11 and 13; STyphi I), a high concentration of *Salmonella* (7.5 ± 0.1 $\log_{10}$ cells mL^{-1}) was measured in effluent samples at the end of the first stabilization period (STyphi I, Table 2). Furthermore, the intestinal microbial balance largely changed compared with STyphi 0, with a significant decrease of bifidobacteria (minus 1.2 $\log_{10}$ unit) and a significant increase of *Enterobacteriaceae, Atopobium* spp. and the *C. coccoides- E. rectale* group. During this period (STyphi I), the *C. coccoides- E. rectale* group became the predominant bacterial group. Addition of amoxicillin (90 mg day^{-1}, ATB I) induced a significant decrease in *Salmonella* concentration (more than 1.5 $\log_{10}$ units) compared with STyphi I (Table 2) and a shift in the different bacterial concentrations, which returned to values not significantly different from STyphi 0 ($P > 0.05$). The only exception was *Enterobacteriaceae* which in contrast to *Salmonella*, did not decrease during ATB I and remained significantly higher than during STyphi 0. During STyphi II with no antibiotic, most bacterial populations (including *Salmonella*) went back to values similar to STyphi I, except for *Bifidobacterium* spp. and *C. coccoides- E. rectale* concentrations which were significantly higher and lower, respectively. The second amoxicillin treatment (180 mg day^{-1}, ATB II) induced similar changes as during ATB I, but the effects on bacterial populations measured during the last three days were significant only for *Salmonella* and the *C. coccoides- E. rectale* group. Finally, during the last stabilization period without antibiotic (STyphi III), bacterial populations went back to values similar to STyphi 0, except for *Salmonella* and total *Enterobacteriaceae* which remained significantly higher than for STyphi 0. No effect of treatments was observed on total bacteria or on *Bacteroides* concentrations during the whole fermentation.

Microbial populations analyzed by FISH-microscopy

Bacterial analyses with FISH coupled to microscopic counts for total bacteria and *S.* Typhimurium used to monitor overtime the fermentation process corroborated data obtained with flow cytometry, with a significant decrease in *Salmonella* concentration during ATB I and II. No protease treatment was applied to these samples. However, a more pronounced inhibition of *S.* Typhimurium was measured with ATB I (minus 1.3 $\log_{10}$ units compared with STyphi I) compared with ATB II (minus 0.95 $\log_{10}$ units compared to STyphi II) (Figure 2). Furthermore cell aggregation was enhanced during antibiotic treatments. Total bacteria counts measured with FISH-microscopy were significantly lower (minus 1.2 $\log_{10}$ units) than by FISH-flow cytometry, decreased from STyphi 0 to STyphi I and remained stable during the subsequent treatments.

Metabolites analyses

The profiles of metabolite concentrations during the different fermentation periods are shown in Figure 3. Acetate was the main metabolite detected during STyphi 0 and throughout the fermentation (40-80 mM), whereas propionate and butyrate were present at low concentrations ($\leq$ 20 mM). Lactate was also detected, although in variable concentrations (1-20 mM), during the whole fermentation. Addition of *S.* Typhimurium induced a significant increase in butyrate (6.0 ± 0.1 and 13.5 ± 1.8 mM for STyphi 0 and STyphi I, respectively), whereas the other metabolites were not modified between the two periods (Table 3). ATB I dramatically decreased total metabolite concentration compared with STyphi I, especially due to a strong decrease in acetate, while formate and lactate increased. During STyphi II, metabolite concentrations went back to their previous levels of STyphi I, except for acetate and butyrate which remained significantly lower than during STyphi I. ATB II induced slightly different metabolite concentration changes compared with ATB I. A less pronounced decrease in acetate concentration was observed, whereas lactate and formate were

significantly more increased. During the last stabilization period (STyphi III) metabolite concentrations returned to their previous levels of STyphi II, except for propionate and formate (Table 3).

Discussion

Recently we successfully developed a new *in vitro* model of intestinal fermentation with immobilized fecal microbiota (Cinquin *et al.*, 2004; Cinquin *et al.*, 2006a; Cinquin *et al.*, 2006b; Cleusix *et al.*, 2008). One major advantage of cell immobilization in intestinal fermentation models is the very high microbial and metabolic stability due to entrapment and growth of fecal microbiota in polysaccharide beads, which was tested over long fermentation periods (up to 7 weeks).

In this study, we used the same approach with fecal sample immobilization and continuous fermentation to develop an original model simulating *Salmonella* gut infection in children. We showed that cell immobilization can circumvent problems due to wash-out of exogenous enteropathogens observed in conventional *in vitro* intestinal fermentation models operated with planktonic cells (Blake *et al.*, 2003; Carman *et al.*, 2004; Payne *et al.*, 2003). According to our initial assumption, addition of *S.* Typhimurium immobilized in polysaccharide beads allowed to recover and maintain *S.* Typhimurium in the reactor effluents during the entire fermentation of 43 days. Seven days after addition of *S.* Typhimurium colonized beads (days 18-20; STyphi I), the strain was detected at high concentrations (7.5 $\log_{10}$ cells mL^{-1}) in effluent samples, in agreement with *in vivo* concentrations of non-typhoid *Salmonella* shedding of up to 10^6-10^7 organisms per gram of faeces measured in some children during early convalescence (Cruickshank & Humphrey, 1987). A daily analysis of *S.* Typhimurium and commensal bacteria was then performed in effluent samples. A protease treatment was used to destroy aggregates before bacterial enumeration with FISH-flow cytometry. However, no sample protease treatment was done before analysis with FISH-microscopy. Therefore the

aggregation phenomena can explain the lower total and *Salmonella* cell counts detected with FISH-microscopy compared to FISH-flow cytometry. Furthermore the higher inhibition effects of antibiotics on *Salmonella* detected with FISH-microscopy compared to FISH-flow cytometry can also be due to enhanced cell aggregation observed during antibiotic treatments. Total bacteria and bifidobacteria populations measured during STyphi 0 and in feces were high and very close. Unfortunately, the other populations could not be determined in feces due to a lack of sample. We showed in previous studies that the main populations of child and adult fecal samples were well preserved during immobilization and long-term continuous fermentation. However, differences of microbial balance between the fecal inoculum and reactor effluents occurred due to changes of environmental conditions between the host intestine and the fermentation model, such as medium composition, pH, and retention time (Cinquin *et al.*, 2004; Cinquin *et al.*, 2006b, Cleusix *et al.*, 2008). Such differences are often observed with *in vitro* intestinal fermentation systems (Macfarlane & Macfarlane, 2007). Furthermore, conditions of the proximal colon applied in the fermentation model are very different to that of the distal colon where composition of fecal material is more akin to feces. Compared with STyphi 0, *S.* Typhimurium colonization (STyphi I) induced a strong modification in the microbial balance. Bifidobacteria, present in high numbers during STyphi 0 were significantly decreased, whereas *C. cocoides- E. rectale* group and *Atopobium* spp. were strongly increased ($P < 0.05$). In contrast to bacterial populations, metabolites were only slightly modified, with only a significant increase in butyrate concentrations during STyphi I. This butyrate accumulation may be explained by an increase in the *C. cocoides- E. rectale* group, which contains most of the butyrate producers (Barcenilla *et al.*, 2000).

As expected, addition of amoxicillin in the reactor significantly reduced *S.* Typhimurium concentration in effluent samples and changed the microbial balance. *C. coccoides- E. rectale* and *Atopobium* populations were strongly inhibited by amoxicillin, whereas *Bacteroides,*

bifidobacteria and total *Enterobacteriaceae* were not affected. Marked decreases in the *C. cocoides- E. rectale* group have already been described by Barc *et al.* (2004) in human fecal flora-associated mice receiving amoxicillin-clavulanic acid (150 mg (kg of body weight)$^{-1}$) for 7 days. The lack of activity of amoxicillin on *Bacteroides-Prevotella* and *Enterobacteriaceae* was also expected because it is known that certain *Bacteroides* such as *Bacteroides fragilis* and some *Enterobacteriaceae* (*Klebsiella* spp., *E. coli*) are not inhibited during amoxicillin treatments in humans (Sullivan *et al.*, 2001; Floor *et al.*, 1994). Indeed, resistance to β-lactams via β-lactamase production has been largely described in *Bacteroides* spp. and *Enterobacteriaceae* (Kader *et al.*, 2004; Papaparaskevas *et al.*, 2005). Unexpectedly, bifidobacteria were not inhibited by amoxicillin treatments and their growth was even stimulated during ATB I. Although it is generally admitted that bifidobacteria are highly sensitive to β-lactams (Moubareck *et al.*, 2005) some strains are resistant to amoxicillin (Lim *et al.*, 1993; Vlková *et al.*, 2006). However, bifidobacteria overgrowth during ATB I may not be directly associated with the effects of antibiotics. It could also be due to the strong decrease in the *C. coccoides- E. rectale* group, which allowed the growth of bifidobacteria that came back to their previous concentrations (STyphi 0). It seems that bifidobacteria were particularly competitive in the system and that they prevented the overgrowth of *Bacteroides* spp. during amoxicillin treatment, contrary to some *in vivo* observations (Christensson et al., 1991). *Enterobacteriaceae* were not significantly decreased during ATB I, and ATB II despite a significant decrease of *Salmonella*, and became the second most dominant group during STyphi II, taking the place of the *C. coccoides- E rectale* group. Such increases in *Enterobacteriaceae* have also been frequently described in the literature after amoxicillin treatments in humans (Christensson *et al.*, 1991; Sullivan *et al.*, 2001).

In parallel to modifications of microbial balance during ATB I and II, metabolite ratios were also strongly modified. ATB I decreased acetate and increased lactate and formate concentrations, whereas butyrate and propionate were not changed compared with STyphi I. Doubling the amoxicillin concentration (ATB II) also led to a similar decrease in acetate concentration compared with STyphi II, but also to twice as much lactate and formate and to a decrease in propionate and butyrate concentrations. Lactate and formate are intermediate metabolites produced by many different bacteria (Cummings & Macfarlane, 1991), further metabolised by other bacteria into CO_2 and major SCFA (acetate, propionate or butyrate) (Duncan *et al.*, 2004; Seeliger *et al.*, 2002). These metabolites generally do not accumulate in the human colon (Bernalier *et al.*, 1999). Their accumulation together with the changes in SCFA ratios during ABT I and II suggest that lactate- and formate-utilizing bacteria were inactivated during amoxicillin treatments. The marked decrease in the *C. cocoides-E. rectale* group, which harbours many lactate-utilizing bacteria (Duncan *et al.*, 2004) is likely responsible for lactate accumulation during these periods.

Immobilization of *S.* Typhimurium and its addition to the recently developed *in vitro* model of intestinal fermentation with immobilized fecal microbiota led to stable high levels of *S.* Typhimurium in the effluent of the continuous gut reactor simulating *Salmonella* shedding observed in certain children. Moreover, immobilization prevented washout of *S.* Typhimurium during antibiotic treatments, which allowed comparison of two treatments during the same fermentation with the same fecal inoculum. The effects of amoxicillin measured with this new *in vitro* colonic fermentation model for *Salmonella* infections are in agreement with *in vivo* observations showing a high disturbance of the intestinal microbiota balance with decreases in the *C. coccoides - E. rectale* group and increases in *Enterobacteriaceae* ratios. Furthermore, metabolic ratios tested in the *in vitro* model correlated with microbial change, providing further validation to this model. Therefore, this

new model is a promising tool for simulating intestinal infections in humans with the aim of developing and testing the effects of different antimicrobials on intestinal enteropathogens as well as commensal bacteria.

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Tables

Table 1 Oligonucleotide probes and hybridization conditions used to target predominant bacterial groups

Probes	Dyes*	Target organisms	Formamide (%)	Temperature (ºC)	Probe references
Sal 3	Cy-3	*Salmonella enterica*	0	45	Nordentoft *et al.*, 1997
NonEub338	Cy-5	NA	30	35	Wallner *et al.*, 1993
Bif 164	Cy-5	*Bifidobacterium*	30	35	Langendijk *et al.*, 1995
Bac 303	Cy-5	*Bacteroides-Prevotella*	30	35	Manz *et al.*, 1996
Erec 482	Cy-5	*C. coccoides-E. rectale*	30	35	Franks *et al.*, 1998
Ato 291	Cy-5	*Atopobium*	30	35	Harmsen *et al.*, 2000
Enter 432	Cy-5	Enterobacteria	30	35	Sghir *et al.*, 2000
Sal 3	Cy-5	*Salmonella*	30	35	Nordentoft *et al.*, 1997

*The oligonucleotide probe labelled at the 5' end with Cy-3 was detected with microscopy whereas Cy-5 labelled probes were used in flow cytometry. DAPI and SYBR Green I-stains were used for total bacteria enumeration in microscopy and flow cytometry, respectively.

Table 2 Bacterial populations in fermentation samples during pseudo-steady states of each treatment period measured by FISH-flow cytometry

	Bacterial counts (log_{10} cells mL^{-1} medium)*					
Bacterial popualtions	STyphi 0	STyphi I	ATB I	STyphi II	ATB II	STyphi III
Bifidobacterium	10.2 ± 0.1c	9.0 ± 0.2a	10.5 ± 0.3c	9.9 ± 0.3bc	9.7 ± 0.5ab	10.3 ± 0.1c
C. coccoides-E. rectale	9.1 ± 0.1bc	10.3 ± 0.3d	8.5 ± 0.1ab	9.5 ± 0.3c	8.4 ± 0.4a	8.5 ± 0.4ab
Bacteroides	8.8 ± 0.2a	9.0 ± 0.2a	9.0 ± 0.5a	9.0 ± 0.2a	8.6 ± 0.1a	9.0 ± 0.02a
Atopobium	7.3 ± 0.04a	9.2 ± 0.1c	7.5 ± 0.4a	8.7 ± 0.3bc	8.0 ± 0.5ab	7.2 ± 0.7a
Enterobacteriaceae	NDc	8.9 ± 0.2b	8.2 ± 0.3ab	8.3 ± 0.2ab	7.4 ± 0.3a	9.4 ± 0.3b
Salmonella	ND	7.5 ± 0.1a	NDb	8.1 ± 0.2a	NDb	7.6 ± 0.2a
Total bacteria	10.3 ± 0.4a	10.4 ± 0.2a	10.5 ± 0.1a	10.3 ± 0.1a	10.0 ± 0.3b	10.4 ± 0.2a

*Data are means ± S.D. for the last 3 days for each fermentation period, $n = 2$. Values with different letters in a row are significantly different with the Tukey's test, $P < 0.05$.

¶ND, not detected, below detection limit of the method (log_{10} 6 cells mL^{-1}).

Table 3 Metabolite concentrations in effluent samples during pseudo-steady states of each treatment period measured by HPLC

	Metabolite concentration (mM)*					
Metabolites	STyphi 0	STyphi I	ATB I	STyphi II	ATB II	STyphi III
Acetate	69.6 ± 2.1bc	72.5 ± 3.6c	45.9 ± 5.1a	63.6 ± 2.7b	50.1 ± 3.7a	64.8 ± 1.7bc
Propionate	5.2 ± 0.5b	6.1 ± 2.0bc	6.2 ± 0.6bc	7.5 ± 1.0c	2.2 ± 0.4a	3.8 ± 0.2ab
Butyrate	6.0 ± 0.1ab	13.5 ± 1.8c	13.3 ± 2.6c	8.2 ± 1.5b	3.9 ± 1.5a	7.9 ± 1.5ab
Formate	NDa[¶]	NDa	3.8 ± 0.8b	NDa	7.2 ± 1.8bc	10.5 ± 1.4c
Lactate	1.3 ± 0.2a	1.2 ± 0.4a	7.9 ± 1.0b	2.0 ± 0.5a	17.1 ± 3.0c	4.1 ± 1.6d
Total metabolites	82.1 ± 1.6abc	93.3 ± 4.0c	77.1 ± 4.2a	81.3 ± 2.4abc	80.5 ± 5.1ab	91.2 ± 3.5bc

*Data are means ± S.D. for the last 3 days for each fermentation period, $n = 2$. Values with different letters in a row are significantly different with the Tukey's test, $P < 0.05$.

[¶]ND, not detected, below detection limit of the method.

Figure legends

Fig. 1. Time schedule of continuous intestinal fermentation experiments during different treatment periods: BC, beads colonization; STyphi 0, stabilization of the system, days 3-10; SB, addition of *S.* Typhimurium M557 beads, days 11 and 13; STyphi I, stabilization of *S.* Typhimurium M557 concentrations in effluent samples, days 14-20; ATB I, daily addition of 90 mg amoxicillin, days 21-25; STyphi II, stabilization period, days 26-34; ATB II, daily addition of 180 mg amoxicillin, days 35-39, STyphi III; stabilization period, days 40-43.

Fig. 2. Total bacteria and *Salmonella* enumerated by FISH coupled with microscopy. DAPI and Cy3 labeled Sal 3-probe were used to detect total bacteria and *Salmonella*, respectively, during the different fermentation periods. STyphi 0, stabilization period I without *Salmonella*. STyphi I, stabilization period II after *Salmonella* addition; ATB I, first antibiotherapy, 90 mg day^{-1}; STyphi II, stabilization period; ATB II, second antibiotherapy, 180 mg day^{-1}; STyphi III, stabilization period. Significance is based on last three days of each period.

Fig. 3. Short chain fatty acids and lactate concentrations in effluent samples during the 43-day continuous fermentation. Total metabolites (♦), acetate(●), butyrate (Δ), propionate (▲), formate (○), lactate (◊). Data are means of triplicate analyses.

Figure 1

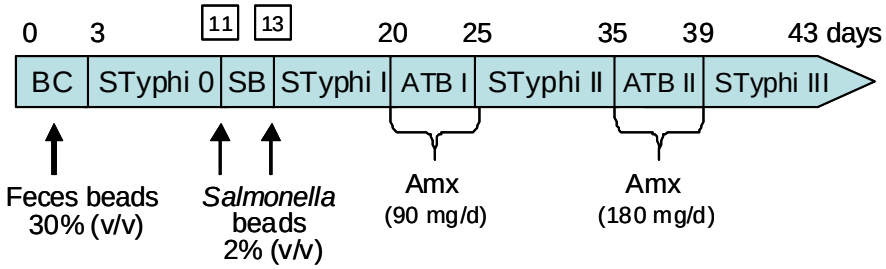

0
3
11
13
20
25
35
39
43 days
BC
STyphi 0
SB
STyphi I
ATB I
STyphi II
ATB II
STyphi III
Feces beads
30% (v/v)
Salmonella
beads
2% (v/v)
Amx
(90 mg/d)
Amx
(180 mg/d)

Figure 2

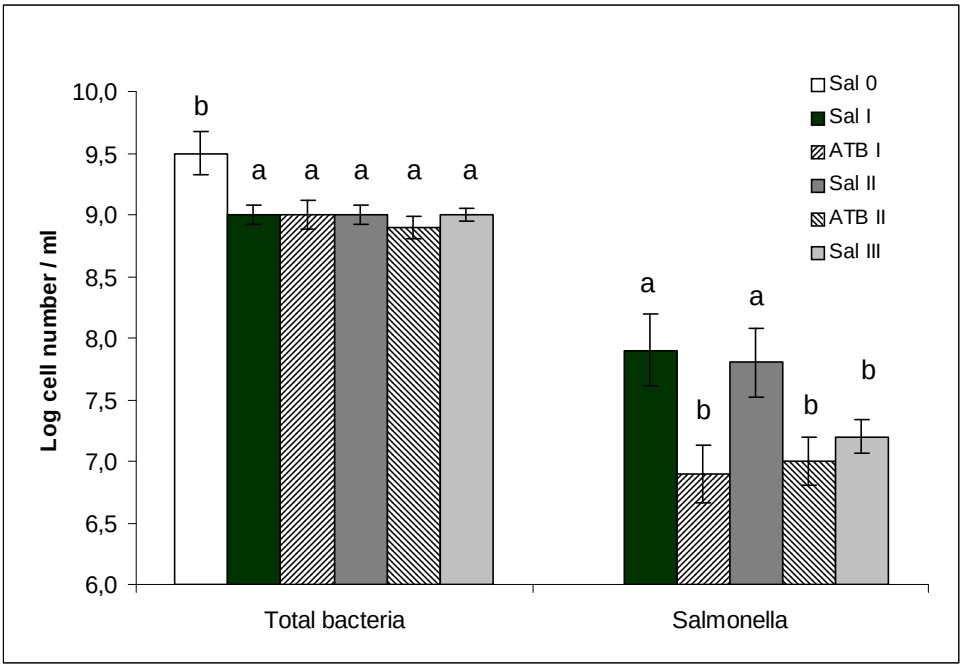
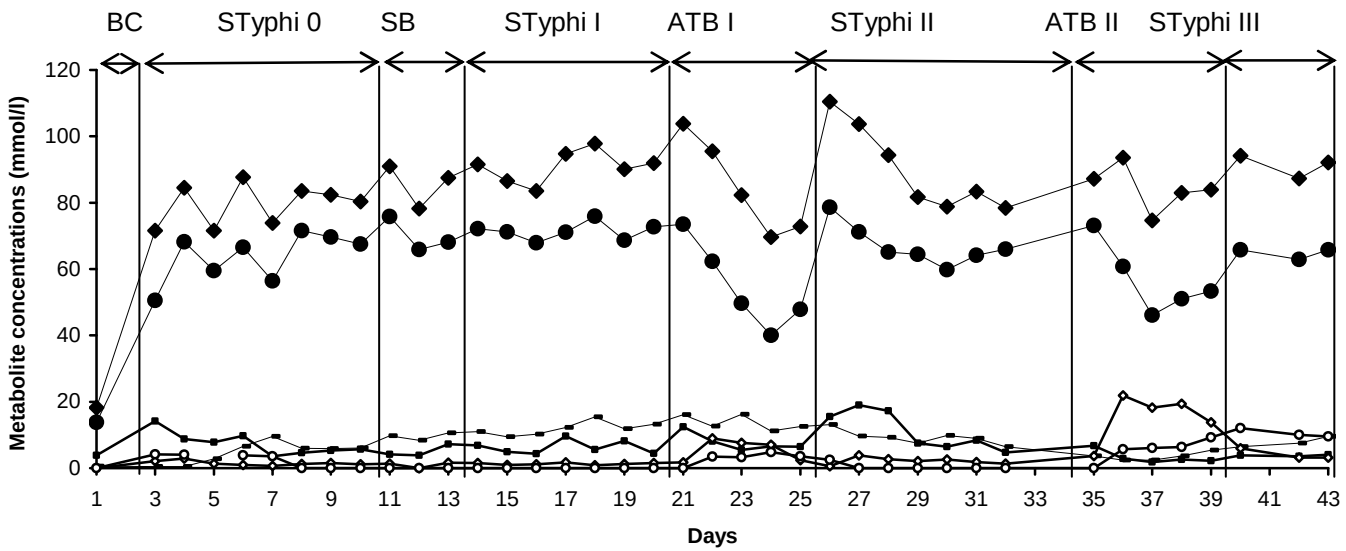

10,0
9,5
9,0
8,5
8,0
7,5
7,0
6,5
6,0
Log cell number / ml
b
a
a
a
a
a
a
b
a
b
b
Sal 0
Sal I
ATB I
Sal II
ATB II
Sal III
Total bacteria
Salmonella

Figure 3


BC
STyphi 0
SB
STyphi I
ATB I
STyphi II
ATB II
STyphi III
Metabolite concentrations (mmol/l)
120
100
80
60
40
20
0
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
33
35
37
39
41
43
Days