New *in vitro* model of *Salmonella* infection in children using immobilized fecal microbiota to compare alternative treatments to antibiotics

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Introduction

Salmonella infections due to microbial contamination by food remain a major issue to be solved. Although salmonellosis is normally self-limiting, this intestinal infection can lead to life-threatening complications, especially in children (below 4 year-old) and in elderly, who have to be treated with effective antibiotics. Because an increasing incidence of *Salmonella* showing multiple drug resistances (MDR) has been observed in recent years, alternative treatments and appropriate models to test their effectiveness are of urgent need. A new *in vitro* continuous colonic fermentation model with immobilized fecal infant microbiota was recently developed and validated (Cinquin, 2006), with several advantages compared to traditional free-cell colonic models (high cell density; preservation of fecal bacterial diversity and metabolic activity, and high stability with the possibility to test several treatments within the same experiment and the same inoculum, etc.). Based on this model, the aim of the present study was to develop and validate a new *in vitro* colonic continuous fermentation model using immobilized fecal microbiota to simulate an intestinal *Salmonella* infection in children and to compare the effects of a conventional antibiotic treatment (amoxicillin [Amx]/clavulanic acid [Clv]) with a promising probiotic, *Bifidobacterium thermophilum* RBL67.

Materials and methods

Fecal sample collection, preparation and immobilization

Fresh feces from a healthy 3 year-old child were collected, prepared and immobilized within 2 h after defecation using a dispersion process in a two-phase system under strictly anaerobic conditions as described by Cinquin (2004). Briefly, a polymer solution composed of gellan gum (2.5%, v/w) and xanthan gum (0.25 %, v/w) dissolved in sodium citrate (0.2 %, v/w) was autoclaved, cooled to 43 °C and inoculated with 2% (v/v) of the fecal inoculum. Beads were formed within pure sunflower oil and hardened by soaking in sterile 0.1 M $CaCl_2$. Beads with a diameter in the 1.0 – 2.0 mm range were selected by wet sieving in sterile 0.1 M $CaCl_2$. A similar process but aseptically under aerobic conditions was used for the immobilization of *S.* Typhimurium M557 sensitive to Amx/Clv and *B. thermophilum* RBL67.

Fermentation procedures

Fecal beads colonization was carried out for 48 h in batch fermentation in two independently operated stirred glass bioreactors with a working volume of 200 mL containing 30% (v/v) of freshly inoculated fecal beads trapped in the reactor. Culture medium was supplied trice and appropriate conditions (T=37 °C; pH=5.7, anaerobiosis) mimicking a child proximal colon (Fallingborg, 1999) were maintained. Continuous fermentation was started by keeping the same fermentation parameters and by adding a stirred feedstock vessel supplying sterile culture medium at a flow rate of 4 mL/h and an effluent receiving vessel connected via peristaltic pumps individually to each reactor (Figure 1). Whereas reactor 1 (R1) was used to study the therapeutic effect of Amx/Clv and *B. thermophilum* RBL67 toward *S.* Typhimurium M557, the prophylactic effect of *B. thermophilum* RBL67 was tested in reactor 2 (R2) (Figure 2).

Bacteriological and metabolite analysis

Concentrations of *S.* Typhimurium M557, total anaerobes and bifidobacteria were monitored daily by plate counts using selective CHROMagar™ Salmonella (Becton Dickinson), Wilkins-Charlgren Agar (Oxoid) and Beerens Agar (Beerens, 1991), respectively. Major intestinal bacterial populations were monitored by fluorescence *in situ* hybridization (FISH) combined with flow cytometry (Cleusix, 2007) using specific Cy5-labelled, 16S rRNA-targeted oligonucleotide probes: Bif 164 for bifidobacteria; Erec 482 for *Clostridium coccoides-Eubacterium rectale* group; Bac 303 for *Bacteroides-Prevotella* group, Enter 1432 for enterobacteria, Ato 291 for *Atopobium* spp. Short-chain fatty acids (SCFA: acetate, propionate, butyrate) were daily analyzed by HPLC.

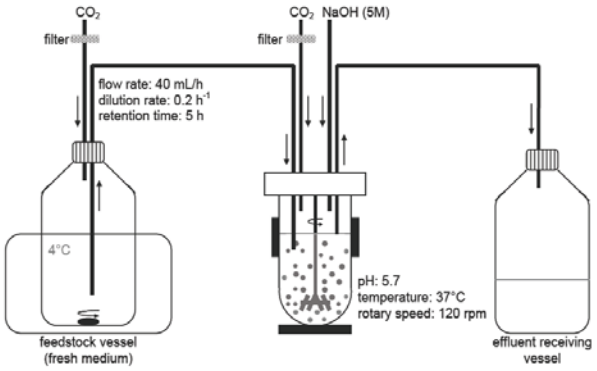

Fig. 1: **Child colonic fermentation**

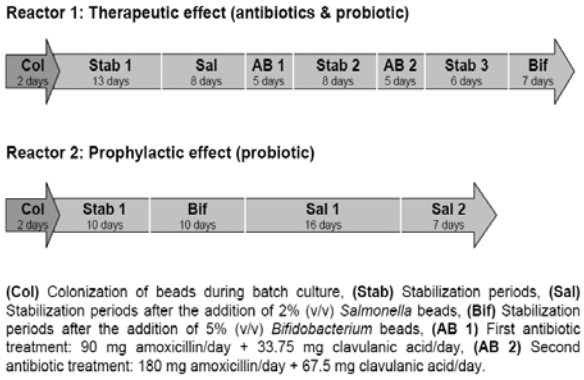

Fig. 2: **Experimental setup**

Results and discussion

Therapeutic effect of amoxicillin/clavulanic acid and B. thermophilum RBL67 on Salmonella infection

At the end of Stab 1 in R1, a high and stable bacterial population was recovered in effluent samples (10.3 – 10.8 Log cell number/mL) with a SCFA profile similar to that of the fecal inoculum. At the end of Sal, after adding 2% (v/v) of *S.* Typhimurium M557 beads (1.4×10^{11} CFU/g beads), *Salmonella* concentrations in efluent samples stabilized at 4.6 ± 0.1 Log CFU/mL (Fig. 3) without modifying the microbial balance (Fig. 5). On the contrary, an impressive disturbance of the bacterial ecosystem was observed during the two periods of antibiotherapy (AB 1 and 2), characterized by different antibiotic concentrations (AB 1: $Amx_{[90\ mg]}/Clv_{[33.75\ mg]}$ and AB 2: $Amx_{[180\ mg]}/Clv_{[67.5\ mg]}$) added for 5 consecutive days in R1. Surprisingly, whereas total bacterial counts decreased significantly (- 1 Log unit), *Salmonella* concentrations increased on the third of both antibiotherapies, to finally reach higher counts than during Sal (Fig. 3). In parallel, a drastic modification of the microbial population and metabolite profiles were observed (Fig. 5). Whereas the *C. coccoides-E. rectale* group was the most abundant (95 %) during Sal and Stab 1, AB 1 and 2 induced a dramatic decrease in *C. coccoides-E. rectale* group accompanied by a conspicuous increase in enterobacteria, *Bacteroides-Prevotella* and *Atopobium* populations. In accordance with the decrease in total bacterial concentration (Fig. 3), total SCFA concentration decreased drastically from 135 ± 8 mM during Sal to 32 ± 3 mM during AB 1 and 2. The SCFA profile was also strongly modified with an increase in propionate and a decrease in butyrate ratios, whereas acetate persisted at the same level (Fig. 6). The bacterial equilibrium reestablished after AB 1 (Stab 2) was persistently modified after AB 2 (Stab 3) with a drastic increase in bifidobacteria concentration in parallel with the decrease of *S.* Thyphimurium M557. Addition of *B. thermophilum* RBL67 (Bif) partially reestablished bacterial and metabolite proportions similar to Stab 1 and 2, and induced a continuous decrease in *Salmonella* counts to a final value of 3.7 Log cell number/mL at the end of Bif (Fig. 3).

Prophylactic effect of B. thermophilum RBL67 on Salmonella infection

In the same way as for R1, R2 was characterized with high and stable bacterial counts over the whole fermentation period. However, in contrary to R1, *S.* Typhimurium M557 added after the supplementation with 5% (v/v) of *B. thermophilum* RBL67 beads (1.3×10^{11} CFU/g beads) was not competitive enough to persistently colonize R2 (Fig. 4). Indeed, *S.* Typhimurium M557 was not detected at the end of Sal 1 and the addition of another 2% (v/v) of *Salmonella* beads did not prevent the wash-out of *S.* Typhimurium M557 (Fig. 4).

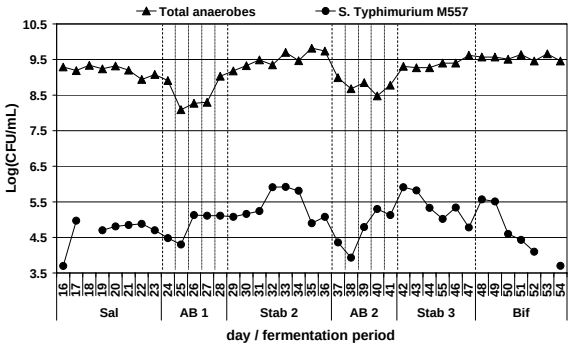

Fig. 3: **Plate counts of *S.* Typhimurium M557 on CHROMagar™ Salmonella in effluent samples of reactor 1.**

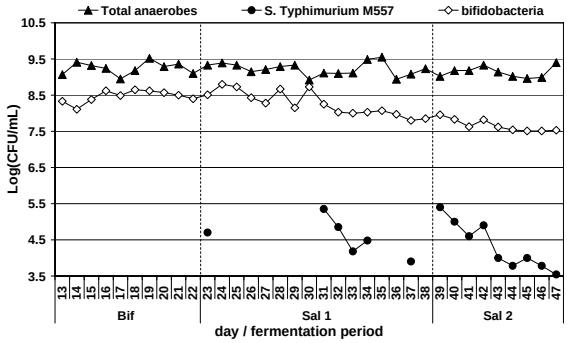

Fig. 4: **Plate counts of S. Typhimurium M557 on CHROMagar™ Salmonella in effluent samples of reactor 2.**

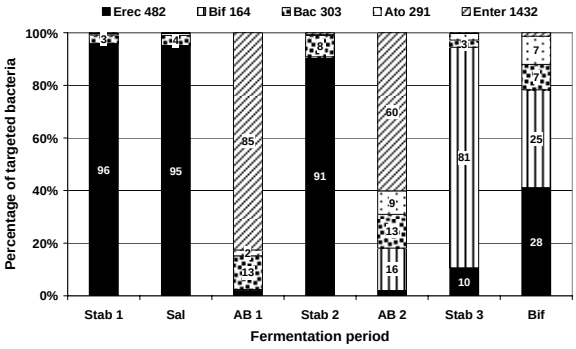

Fig. 5: **Percentage of targeted bacteria measured by FISH combined with flow cytometry (FC) in effluent samples of reactor 1 (reported data are means of three samples harvested during pseudo-steady state of each period).**

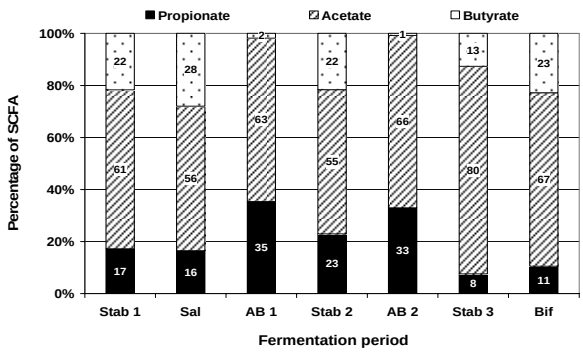

Fig 6: **Percentage of short-chain fatty acids (SCFA: propionate, acetate and butyrate) measured by HPLC analysis in effluent samples of reactor 1 (reported data are means of three samples harvested during pseudo-steady sate of each period).**

The human intestinal bacterial ecosystem is subjected to variations according to diet and environment. Antibiotherapy can disrupt it, leading to a loss of the barrier effect (Sullivan, 2001). In order to study this complex ecosystem, molecular techniques such as FISH combined with flow cytometry are indispensable, since they provide specific identification of uncultivable bacteria. In the present study, and in accordance with a previous study evaluating the effect of Amx/Clv on human-microbiota-associated mice using FISH combined with flow cytometry (Barc, 2004), Amx/Clv led to a high disruption of the dominant bacterial groups with a decrease in *C. coccoides- E. rectale* concomitant to an increase in *Enterobacteriaceae* and *Bacteroides*. Moreover, Amx/Clv did not persistently inhibit *S.* Typhimurium M557, although this strain was sensitive to Amx/Clv in pure culture. Several studies have shown the contrary effect of antibiotics on *Salmonella* infections. They lead to a preliminary extension of clinical symptoms with a tendency to prolong the carrier state

(D'Aoust, 1991). Our results confirmed this negative effect of antibiotherapy on *Salmonella* infection, showing the validity of our model. Moreover, *B. thermophilum* RBL67 prevented the colonization of this *in vitro* intestinal ecosystem with *S.* Thyphimurium M557 added at a very high concentration in R2 and favored its eradication from R1 after antibiotherapy.

Conclusions

Our results showed that the addition of beads colonized with *Salmonella* in an *in vitro* model of intestinal fermentation with immobilized feces can be used to accurately simulate intestinal infection over long time period. Moroeover, this model allowed to investigate the dynamic effects of different treatments on *Salmonella* concentration during the same experiment with the same fecal inoculum. This experiment confirms the inefficacy of antibiotherapy against *S.* Thyphimurium M557, which prolonged the carrier state. On contrary, bifidobacteria and especially *B. thermophilum* RBL67 seemed to be play an important role in fighting salmonella infections.

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