RESEARCH ARTICLE

Glycerol induces reuterin production and decreases *Escherichia coli* population in an *in vitro* model of colonic fermentation with immobilized human feces

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in vitro fermentation model; immobilized intestinal microbiota; *Lactobacillus reuteri* ATCC 55730; reuterin; glycerol.

Abstract

Lactobacillus reuteri ATCC 55730 is a probiotic strain that produces, in the presence of glycerol, reuterin, a broad-spectrum antimicrobial substance. This strain has been shown to prevent intestinal infections *in vivo*; however, its mechanisms of action, and more specifically whether reuterin production occurs within the intestinal tract, are not known. In this study, the effects of *L. reuteri* ATCC 55730 on intestinal microbiota and its capacity to secrete reuterin from glycerol in a novel *in vitro* colonic fermentation model were tested. Two reactors were inoculated with adult immobilized fecal microbiota and the effects of daily addition of *L. reuteri* into one of the reactors ($c.10^8$ CFU mL^{-1}) without or with glycerol were tested on major bacterial populations and compared with addition of glycerol or reuterin alone. The addition of glycerol alone or with *L. reuteri* increased numbers of the *Lactobacillus–Enterococcus* group and decreased *Escherichia coli*. The addition of reuterin significantly and selectively decreased *E. coli* without affecting other bacterial populations. The observed decrease in *E. coli* concentration during the addition of glycerol (in presence or absence of *L. reuteri*) could be due to *in situ* reuterin production because 1,3-propanediol, a typical product of glycerol fermentation, was detected during the addition of glycerol.

Introduction

A healthy gastrointestinal tract is colonized by a highly complex bacterial community, which plays a major role in human health and nutrition. This bacterial community is notably involved in the protection of the gastrointestinal tract by acting as a barrier against colonization by pathogenic bacteria. Among commensal bacteria, lactobacilli and bifidobacteria, including those ingested as probiotics have attracted much attention for their potential to treat or prevent intestinal infections.

Lactobacillus reuteri ATCC 55730 (also designated as SD2112) is a well-known obligate heterofermentative probiotic, isolated from human breast milk, and widely used as a food additive to improve human gastrointestinal health (Casas & Dobrogosz, 2000). *Lactobacillus reuteri* ATCC 55730 can survive low pH as it passes through the stomach to reach the human intestine and colonize it transiently. Clinical trials have shown that the administration of *L. reuteri* ATCC 55730 was associated with therapeutic effects. It reduces the duration of watery diarrhea caused by rotavirus or bacteria (*Salmonella*, *Shigella* and *Campylobacter* species) in children (Weizman *et al*., 2005), and the occurrence of respiratory and gastrointestinal diseases in healthy adults (Tubelius *et al*., 2005). Other *L. reuteri* strains have also been shown to protect against enteropathogens in mice, monkeys, chickens and turkeys (Casas & Dobrogosz, 2000).

Different mechanisms have been proposed to account for the protective action of probiotic bacteria, and more especially *L. reuteri* toward enteropathogens, such as stimulation of the immune system, competition-exclusion, or production of antimicrobial compounds such as organic acids or reuterin. *Lactobacillus reuteri* is known to produce reuterin (3-hydroxypropionaldehyde, 3-HPA), a broad-spectrum antimicrobial substance active in a wide range of pH values against Gram-positive and Gram-negative bacteria (especially *Escherichia coli*), yeasts, fungi, protozoa and viruses (Vollenweider & Lacroix, 2004). Reuterin production and

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accumulation occur during the anaerobic growth of *L. reuteri* in the presence of glycerol and low concentrations of glucose, under conditions of pH and reduction potential found in the small and large intestines (Chung *et al*., 1989). Indeed, reuterin is believed to provide an ecological competitive advantage to *L. reuteri* and to be involved in colonization resistance against potential pathogens (Edens *et al*., 1997). Reuterin synthesis in the colon would occur when sufficient amounts of glycerol become available as a product of luminal microbial fermentations, digestion of luminal fats, sloughed mucus and desquamated epithelial cells, and intestinal clearing of endogenous plasma glycerol (Casas & Dobrogosz, 2000). Very little information is available on the production and/or availability of glycerol for reuterin synthesis in the colon. Moreover, reuterin is very difficult to quantify *in vivo* due to its aldehyde function that reacts with amino and sulfhydryl groups readily available in the intestine (Casas & Dobrogosz, 2000).

In this study, the capacity of *L. reuteri* ATCC 55730 to produce reuterin from glycerol in a complex bacterial ecosystem similar to that of the human intestine was tested. To estimate reuterin production, two specific markers were analysed: 1,3-propanediol, a typical product of glycerol fermentation formed from reuterin reduction (Biebl *et al*., 1999), and *Escherichia coli* highly sensitive to reuterin activity (Rasch, 2002). With this objective, pure reuterin and *L. reuteri* ATCC 55730 (in absence or presence of glycerol) were added in an *in vitro* model of colonic fermentation with immobilized fecal microbiota recently developed and validated (Cinquin *et al*., 2004, 2006). Immobilization was shown to preserve bacterial diversity and induce high cell density in beads and fermentation effluents as a result of immobilized fecal microbiota growth, cell release from beads and possibly growth of free cells in the bulk medium. Moreover, a very high stability renders this model particularly suitable for long-term fermentation studies and comparative testing of different conditions with the same microbiota in the same experiment (Cinquin *et al*., 2006).

Materials and methods

Bacterial strain and reuterin production

Lactobacillus reuteri ATCC 55730 was used for reuterin production and fermentation experiments. It was routinely cultivated in Mann, Rogosa and Sharpe (MRS) aerobically at 37 °C for 14 h. Reuterin was purified as described by Vollenweider *et al*. (2003). The molarity of the reuterin solution was measured by the assay described for 3-HPA analysis, based on the colorimetric method of Circle *et al*. (1945). Reuterin was stored at 4 °C.

Feces collection and preparation of the fecal inoculum

A fecal sample (46.5 g) was collected from a healthy, nonmethanogenic human adult who had not received antibiotic therapy for the last 3 months. Promptly after defecation, the sample was diluted with prewarmed (37 °C) prereduced (0.05% L-cystein) peptone water (0.1%, pH 7) to obtain a final fecal concentration of 20% (w/v). The suspension was then homogenized in a Stomacher® (Seward, Norfolk, UK) for 2 min and filtered through a 150 μm nylon membrane (Sefar AG, Rüschlikon, Switzerland) to remove large particles, under continuous CO_2 bubbling. When about 10 mL of the slurry were filtered, the filtrate was immediately transferred to an anaerobic chamber (Coy Laboratories, Ann Arbor, MI) with a nitrogen atmosphere containing 5% (v/v) hydrogen, for immobilization in polysaccharide beads.

Immobilization technique

The immobilization procedure in 1–2 mm polysaccharide gel beads made of gellan (2.5% w/v) and xanthan gum (0.25% w/v) was based on a dispersion process in a two-phase system, as already described (Cinquin *et al*., 2006). The entire process was completed in anaerobic conditions within 1 h.

Culture medium

The culture medium used to simulate adult human ileal chyme was the same as previously validated by Macfarlane *et al*. (1998) with one modification. The reducing agent (L-cystein) was replaced by titanium (III) citrate (0.6 mM) to avoid binding of L-cystein sulfhydryl groups with reuterin. A vitamin solution described by Gibson & Wang (1994) 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) culture medium. Depending on the test period, this medium was supplemented with different glycerol concentrations (10 or 100 mM) or with 1.3 mM of pure reuterin.

Fermentation procedures

Duplicate single-stage reactors based on the model described by Cinquin *et al*. (2004) were used to mimic the microbial ecosystem of the adult proximal colon. Two stirred glass reactors with a volume of 300 mL (Sixfors, Infors, Bottmingen, Switzerland) were filled with 210 mL culture medium and 90 mL freshly inoculated beads. Batch fermentations were first carried out for bead colonization (BC, days 1 and 2). During colonization, the fermented medium was aseptically replaced by fresh culture medium every 12 h. Temperature (37 °C) was automatically

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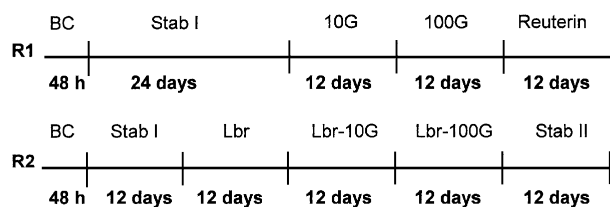

Fig. 1. Time schedule of continuous intestinal fermentation experiments during different treatment periods in reactors 1 and 2. BC, bead colonization; Stab I, stabilization I; Lbr, *Lactobacillus reuteri*; Lbr-10G, *Lb. reuteri* and 10 mM glycerol; Lbr-100G, *Lb. reuteri* and 100 mM glycerol; Stab II, stabilization II; 10G, 10 mM glycerol; 100G, 100 mM glycerol.

controlled, and pH was maintained at 5.7 by addition of NaOH at 5 M. Anaerobic conditions were maintained during the whole fermentation by a continuous flow of pure CO_2 in the headspace.

After bead colonization, each reactor was connected to a stirred feedstock vessel containing the sterile culture medium at 4 °C under CO_2 atmosphere and to an effluent-receiving vessel. Continuous feeding and harvesting of medium was carried out using peristaltic pumps (Reglo analog, Ismatec, Glattbrugg, Switzerland). Beads were kept in the reactors using pinched stainless-steel harvesting tubes. The mean retention time of the medium was set at 25 h by adjusting the feed flow rate to 12 mL h^{-1} and the pH was set at 5.7 (Cummings & Macfarlane, 1991). These set points were chosen to simulate the conditions of the adult proximal colon.

The culture was carried out for a total of 62 days (2 days of batch culture, plus 60 days of continuous culture) and was divided into different test periods of 12–24 days (Fig. 1). The first reactor (R1) was used to test the effects of glycerol addition (final concentration of 10 [10G] or 100 [100G] mM) and purified reuterin (final concentration of 1.3 mM) on bacterial populations and metabolic activities, whereas the second reactor (R2) was used to test the effects of *L. reuteri* ATCC 55730 (added daily at a final concentration of *c.*10^8 CFU mL^{-1} into the reactor) in the presence (final concentration of 10 [Lbr-10G] or 100 [Lbr-100G] mM) or absence of glycerol (Lbr). Before R2 inoculation, *L. reuteri* ATCC 55730 was grown overnight at 37 °C for 14 h in 200 mL MRS broth. The culture was then centrifuged (10 000 ***g*** for 15 min at 4 °C) and the pellet resuspended in 10 mL (reaching *c.* 3×10^{10} CFU mL^{-1}) of culture medium. The cell suspension was then added to the reactor under continuous mixing. Effluent samples (15 mL) were collected daily from both reactors for plate counts, metabolites [short-chain fatty acids (SCFA), lactate, glycerol, reuterin and 1,3-propanediol] and FISH analyses. Sampling of R2 was carried out 5 h after *L. reuteri* ATCC 55730 introduction. 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).

Bacterial enumeration by plate counts

The concentration of *L. reuteri* ATCC 55730 in pure culture was determined by plate counts on MRS agar. *Lactobacillus reuteri* ATCC 55730 was recently shown to be resistant to tetracycline (Kastner *et al.*, 2006). The LAMVAB agar, selective for isolating lactobacilli in feces (Hartemink *et al.*, 1997), and *Lactobacillus* Anaerobic MRS with Vancomycin and Bromocresol green (LAMVAB) agar supplemented with tetracycline were used to enumerate total lactobacilli and *L. reuteri* ATCC 55730, respectively, in fecal and intestinal fermentation samples. In a first attempt to selectively enumerate *L. reuteri* ATCC 55730, a concentration of 200 μg mL^{-1} tetracycline was added to LAMVAB medium. This tetracycline concentration was chosen after preliminary tests with pure cultures of *L. reuteri* ATCC 55730 and other *Lactobacillus* spp. commonly encountered in human feces. Spiked fecal samples with the same lactobacilli cultures were also tested (data not shown). Agar plates were incubated in anaerobic jars at 37 °C for up to 5 days. Results were expressed as log CFU mL^{-1} of effluent samples. The detection limit of the method was determined to be log 4.

Bacterial enumeration by FISH

FISH hybridization analyses were performed as described by Cinquin *et al.* (2006) on fixed fecal inoculum, fermentation samples (1.5 mL) and beads, from the last 3 days and last day of each pseudo-steady-state period, respectively. Beads were dissolved in 1% EDTA solution (pH 7) using a Stomacher® for 5 min. Different 5′ Cy3-labelled 16S rRNA oligonucleotide probes were used with hybridization conditions specific for each probe: Bif164 for *Bifidobacterium* spp. (Langendijk *et al.*, 1995); Bac303 for *Bacteroides–Prevotella* cluster (Manz *et al.*, 1996); Lab158 for *Lactobacillus* and *Enterococcus* (Harmsen *et al.*, 1999); EC1531 for *Escherichia coli* (Poulsen *et al.*, 1995); Erec482 for *Clostridium coccoides–Eubacterium rectale* group (Franks *et al.*, 1998); and Lbre for *Lactobacillus reuteri* (Vogel *et al.*, 1994). For total cell counts 4′, 6-diamidino-2-phenylindole (DAPI) was added at a final concentration of 1 μg mL^{-1}. To prevent fading of fluorescence, citifluor AF1 (Citifluor Ltd, London, UK) was used. Cells were counted visually using an Olympus BX 60 epifluorescence microscope (Olympus Schweiz AG, Volketswil, Switzerland) on 10-well slides (Fisher Scientific SA, Wohlen, Switzerland). To minimize the counting error due to the radial distribution of bacteria in wells, bacterial concentrations were calculated from the bacterial density corresponding to 15 annular regions as already described by Cinquin *et al.* (2006). Each assay was carried out in duplicate. Results were expressed as log cells mL^{-1} of effluent samples or g^{-1} of gel beads or feces. The detection limit of the method was log 6.0 cells mL^{-1} or g^{-1} of fermentation effluents and feces, respectively, and log 7.5 cells g^{-1} of gel beads.

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Table 1. Bacterial populations in fecal inoculum, beads and fermentation samples during the first stabilization period of the continuous cultures measured by FISH

Target organisms	Feces*	Inoculum†	Fermentation‡	Stab I beads§	PCS¶
Total bacteria	10.6–11.6	11.2 ± 0.4	10.2 ± 0.1	10.7 ± 0.2	8.9–10.0
Cl. coccoides–E. rectale	9.9–11.1	10.0 ± 0.3	9.1 ± 0.1	9.1 ± 0.2	9.3–9.5
Escherichia coli	8.0–9.8	9.1 ± 0.3	8.8 ± 0.1	8.9 ± 0.1	7.3
Bifidobacterium spp.	7.2–10.2	9.6 ± 0.3	6.8 ± 0.2	8.6 ± 0.1	< 6.0–9.0
Bacteroides–Prevotella	9.2–10.3	9.2 ± 0.3	7.5 ± 1.0	8.0 ± 0.2	6.8–10.1
Lactobacillus–Enterococcus	8.6–9.5	7.8 ± 0.1	7.5 ± 0.0	8.8 ± 0.2	< 6.0–8.3

*Fecal populations (log cells g^{-1}) of healthy adults reported in various studies (Franks *et al*., 1998; Harmsen *et al*., 1999).
†Bacterial concentrations (log cells g^{-1} of feces) are means ± SD of duplicate analyses of fecal inoculum used in R1 and R2, $n = 2$.
‡Bacterial concentrations (log cells mL^{-1}) are means ± SD of effluent samples harvested during the last 3 days of Stab I in R1 and R2, $n = 6$.
§Bacterial concentrations (log cells g^{-1} of gel) are means ± SD of bead samples harvested during the last day of Stab I in R1 and R2, $n = 2$.
¶Bacterial populations (log cells mL^{-1}) reported for *in vitro* continuous fermentation samples (PCS, proximal colon simulation) by Brück *et al*. (2002), Probert & Gibson (2004) and Child *et al*. (2006) (converted from percentage).

Metabolite analyses

SCFA (acetate, propionate, butyrate), lactate, reuterin, glycerol and 1,3-propanediol were determined by HPLC analysis (Hitachi LaChrome, Merck, Dietikon, Switzerland). Fermentation samples were centrifuged (10 000 ***g***) for 10 min at 4 °C, and supernatants were deep-frozen at − 80 °C. Before analysis, samples (1 mL) were defrosted, diluted in ultra pure water (Millipore AG, Volketswil, Switzerland) and filtered through a 4 mm Titan HPLC filter with a 0.45 µm nylon membrane (Infochroma AG, Zug, Switzerland) directly in vials, which were immediately sealed and analyzed. The analysis was performed at a flow rate of 0.4 mL min^{-1} at 25 °C, with 10 mM H_2SO_4 as eluent and an injection volume of 40 µL. Each analysis was performed in duplicate. The mean metabolite concentrations were expressed in millimolar.

Statistical analyses

A one-way ANOVA was performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL) 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 $P < 0.05$. Values in the text are means ± SD.

Results

Bacterial diversity during the first stabilization period (Stab I) in both reactors

The mean concentrations of the different bacterial populations measured by FISH in fecal inoculum, beads and effluent samples from both reactors at the end of Stab I are shown in Table 1. Using a fecal inoculum of log 11.2 cells g^{-1}, effluent samples and gel beads were highly colonized with 10.2 log cells mL^{-1} and 10.7 log cells g^{-1} at the end of Stab I. Changes in microbial balance were observed in effluent samples and gel beads compared with the inoculum. Bifidobacteria (especially in effluents) and *Bacteroides–Prevotella* concentrations decreased and lactobacilli counts (in beads only) increased. *Clostridium coccoides–Eubacterium rectale* remained the dominant group in all samples. All major groups of intestinal bacteria found in the fecal inoculum were present in effluent samples and reached stable and similar values for R1 and R2 after 9 days of fermentation (Table 2).

Effects of glycerol, reuterin and *L. reuteri* ATCC 55730 on intestinal microbiota composition

In R1, the addition of glycerol did not significantly modify the main bacterial population counts in the effluent samples (Table 2), except for the *Lactobacillus–Enterococcus* group and *E. coli* population. Compared with Stab I, the *Lactobacillus–Enterococcus* group increased significantly by 0.6 log cells mL^{-1} during 100G. However, no increase in commensal *L. reuteri* or in lactobacilli were observed with the Lbre probe (Table 2) or by selective plating (Fig. 2), respectively. During 10G and 100G, *E. coli* decreased by 0.5 and 0.4 log cells mL^{-1}, respectively, compared with Stab I. This effect was only statistically significant ($P < 0.05$) for 10G. The same effect was observed during the addition of reuterin at very low concentrations (1.3 mM).

As expected, Lbr, Lbr-10G and Lbr-100G in R2 significantly increased *Lactobacillus–Enterococcus* and *L. reuteri* counts compared with Stab I (Table 2). When the addition of *L. reuteri* ATCC 55730 was stopped during the second stabilization period (Stab II), *Lactobacillus–Enterococcus* and *L. reuteri* concentrations returned to previous values measured during Stab I (Table 2). Similar data were observed with selective plate counts for total lactobacilli and *L. reuteri* ATCC 55730 on LAMVAB and LAMVAB plus tetracycline, respectively (Fig. 2). Only a fraction of *L. reuteri* ATCC 55730 could be detected with the Lbre oligonucleotide probe in pure culture (data not shown). Moreover, only a small

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Table 2. Bacterial populations measured by FISH in effluent samples from reactors 1 and 2 during pseudo-steady states for different periods of continuous cultures

	Bacterial counts (log cells mL^{-1} medium)*				
Target organisms	Stab I	10G	100G	Reuterin	
Reactor 1					
Total bacteria	10.3 ± 0.3a	10.3 ± 0.1a	10.3 ± 0.1a	10.1 ± 0.2a	
Cl. coccoides–E. rectale	9.0 ± 0.1a	9.0 ± 0.1a	8.9 ± 0.2a	8.8 ± 0.1a	
Escherichia coli	8.6 ± 0.2a	8.1 ± 0.2b	8.2 ± 0.1ab	8.0 ± 0.1b	
Bifidobacterium spp.	6.7 ± 0.3a	6.3 ± 0.1a	6.1 ± 0.1a	6.3 ± 0.2a	
Bacteroides–Prevotella	7.8 ± 0.2a	7.6 ± 0.1a	7.6 ± 0.4a	7.4 ± 0.1a	
Lactobacillus–Enterococcus	7.4 ± 0.1a	7.4 ± 0.2a	8.0 ± 0.1b	7.5 ± 0.1a	
L. reuteri	6.4 ± 0.2a	6.4 ± 0.3a	6.5 ± 0.2a	6.2 ± 0.3a	
	Stab I	Lbr	Lbr-10G	Lbr-100G	Stab II
Reactor 2					
Total bacteria	10.2 ± 0.1a	10.1 ± 0.1a	10.2 ± 0.2a	10.2 ± 0.1a	10.2 ± 0.1a
Cl. coccoides–E. rectale	9.0 ± 0.3a	8.5 ± 0.1a	8.6 ± 0.1a	8.9 ± 0.2a	8.8 ± 0.1a
Escherichia coli	8.8 ± 0.2a	8.9 ± 0.1a	8.3 ± 0.1b	8.3 ± 0.1b	8.5 ± 0.1a
Bifidobacterium spp.	7.0 ± 0.2ab	6.9 ± 0.1ab	7.4 ± 0.1a	7.3 ± 0.1a	6.4 ± 0.4b
Bacteroides–Prevotella	7.2 ± 0.1a	7.4 ± 0.1a	7.5 ± 0.3a	7.5 ± 0.1a	7.6 ± 0.4a
Lactobacillus–Enterococcus	7.5 ± 0.2a	8.5 ± 0.1b	8.3 ± 0.1b	8.2 ± 0.1b	7.6 ± 0.1a
L. reuteri	6.5 ± 0.1a	7.1 ± 0.2b	7.3 ± 0.2b	7.3 ± 0.1b	6.8 ± 0.1a

*Data are means ± SD for the last 3 days for each fermentation period in reactors 1 and 2, $n = 3$. Values with different letters in a row are significantly different by Tukey's test, $P < 0.05$. Stab I, stabilization I; Lbr, *L. reuteri*; Lbr-10G, *L. reuteri* and 10 mM glycerol; Lbr-100G, *L. reuteri* and 100 mM glycerol; Stab II, stabilization II; 10G, 10 mM glycerol; 100G, 100 mM glycerol.

fraction ($c.10^5$ CFU mL^{-1}) of added *L. reuteri* ATCC 55730 (Lbr period) was measured with plate counts on selective LAMVAB medium supplemented with 200 μg tetracycline (Fig. 2, days 14–29), whereas a large increase of total lactobacilli, from 6.5 to 8.6 log CFU mL^{-1}, was measured on LAMVAB medium. The strain became apparently more sensitive to tetracycline in the reactor effluents compared with the pure culture tested during preliminary experiments. Therefore the concentration of tetracycline was decreased to 150 μg mL^{-1} for the rest of the fermentation to improve *L. reuteri* ATCC 55730 detection (Fig. 2, days 30–60). As already observed during 100G, the *E. coli* population selectively and significantly decreased (by 0.5 log cells mL^{-1}) during Lbr-10G and Lbr-100G compared with Stab I, whereas Lbr did not have any detectable effect (Table 2). Moreover, when glycerol addition was stopped in Stab II, bifidobacteria concentrations significantly decreased by 0.9 log cells mL^{-1} compared with Lbr-10G and Lbr-100G.

Effects of glycerol, reuterin and *L. reuteri* ATCC 55730 on metabolic activities

SCFA, glycerol, reuterin and 1,3-propanediol concentrations were analyzed in fermentation samples from both reactors for the last 3 days of each treatment period corresponding to a pseudo-steady state. Total SCFA concentrations ranged from 146 to 174 mM (Table 3) and lactate was only detected in very small amounts in both reactors (< 0.5 mM, data not shown) for all tested conditions.

In R1, 10G did not affect bacterial metabolism compared with Stab I. However, an increase in glycerol concentration during 100G slightly (not significant) decreased the total SCFA concentration and increased butyrate, while decreasing acetate concentrations. This led to a significant increase in butyrate (23.6% vs. 31.1%, $P < 0.05$) and decrease in acetate (49.3% vs. 44.7%, $P < 0.05$) ratios in the total SCFA. The addition of pure reuterin at low concentration (1.3 mM) did not modify the SCFA profile compared with Stab I, but slightly decreased ($P < 0.05$) the total SCFA concentration (Table 3).

Lbr and Lbr-10G in R2 induced a significant increase in total SCFA concentrations and a modification of SCFA profiles due to an increase in acetate and propionate and a decrease in butyrate concentrations compared with Stab I (Table 3). Compared with Lbr and Lbr-10G, Lbr-100G decreased the total SCFA concentration due to a considerable decrease in acetate, while propionate concentration was stable and butyrate increased. SCFA concentration and ratios obtained during Lbr-100G were very similar to those obtained during 100G.

The mean ammonia concentration varied significantly in both reactors for the different treatments (Table 3). Compared with Stab I, 10G and 100G resulted in a decrease in

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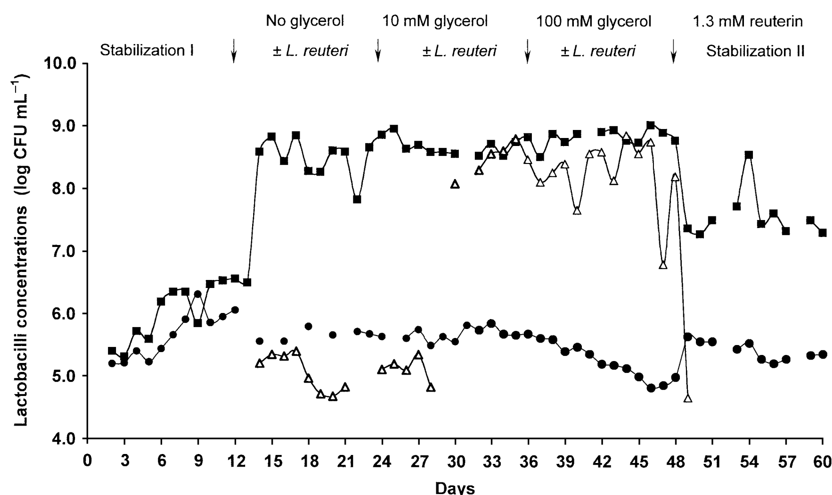

Fig. 2. Effects of glycerol, *Lactobacillus reuteri* ATCC 55730 and reuterin addition on total lactobacilli (enumerated on LAMVAB agar plates) and *L. reuteri* ATCC 55730 (LAMVAB supplemented with tetracycline at 200 μg mL^{-1} until day 30 and 150 μg mL^{-1} thereafter) populations during continuous fermentations in reactors 1 and 2: lactobacilli in R1 (–•–), lactobacilli in R2 (–■–), *L. reuteri* ATCC 55730 in R2 (–△–).

Table 3. Concentrations of total SCFA, ammonia, and molar proportions of acetate, propionate and butyrate measured in effluent samples during different treatments*

	Fermentation conditions							
	Stab I		10G		100G		Reuterin	
	(mM)	(%)	(mM)	(%)	(mM)	(%)	(mM)	(%)
Reactor 1								
Total SCFA†	158.7a		161.9a		153.0a		147.1b	
Acetate	79.1 ± 5.1a	49.8	79.8 ± 3.2a	49.3	68.3 ± 4.9a	44.7	75.4 ± 2.5a	51.2
Propionate	41.6 ± 2.3ab	26.2	43.8 ± 1.4ab	27.1	37.0 ± 2.9a	24.2	47.2 ± 7.5b	32.0
Butyrate	38.0a ± 3.6a	24.0	38.2 ± 1.1a	23.6	47.6 ± 3.1b	31.1	24.6 ± 3.4c	16.7
Ammonia	84.4a		74.7ab		70.4b		73.4b	

	Stab I		Lbr		Lbr-10G		Lbr-100G		Stab II	
	(mM)	(%)	(mM)	(%)	(mM)	(%)	(mM)	(%)	(mM)	(%)
Reactor 2										
Total SCFA†	146.3a		173.9b		167.5b		157.1a		157.8ab	
Acetate	77.2 ± 3.6ac	52.8	113.8 ± 4.1b	65.4	105.7 ± 0.6b	63.1	74.6 ± 5.0c	47.5	93.8 ± 7.7a	57.7
Propionate	20.9 ± 1.8a	14.2	39.2b ± 2.5b	22.6	40.8 ± 1.5b	24.3	42.1 ± 3.8b	26.8	44.0 ± 2.9b	27.1
Butyrate	48.3 ± 3.0a	33.0	21.0b ± 1.2b	12.0	21.1 ± 2.2b	12.6	40.4 ± 6.4a	25.7	24.7 ± 2.2b	15.2
Ammonia	68.0a		94.5b		83.8c		79.1c		93.7b	

*Data are means ± SD of the last 3 days of each pseudo-steady-state period in reactors 1 and 2, $n = 3$. Values with different letters in a row are significantly different by Tukey's test, $P < 0.05$. Stab I, stabilization I; Lbr, *L. reuteri*; Lbr-10G, *L. reuteri* and 10 mM glycerol; Lbr-100G, *L. reuteri* and 100 mM glycerol; Stab II, stabilization II; 10G, 10 mM glycerol; 100G, 100 mM glycerol.

†Defined as the sum of acetate, propionate and butyrate concentrations.

ammonia concentration in R1, but this effect was only significant ($P < 0.05$) for 100G. During the addition of pure reuterin, an ammonia concentration similar to 10G and 100G, but significantly lower than in Stab I ($P < 0.05$) was measured. In R2, Lbr significantly increased the ammonia concentration compared with Stab I, but this increase was reduced during Lbr-10G and Lbr-100G. During Stab II, the ammonia concentration increased again to reach levels similar to Lbr.

The glycerol concentration in both reactors remained under the detection limit of the HPLC method during the whole fermentation. The concentration of 1,3-propanediol was also under the detection limit of the method (2 mM), except during 100G and Lbr-100G, which induced a

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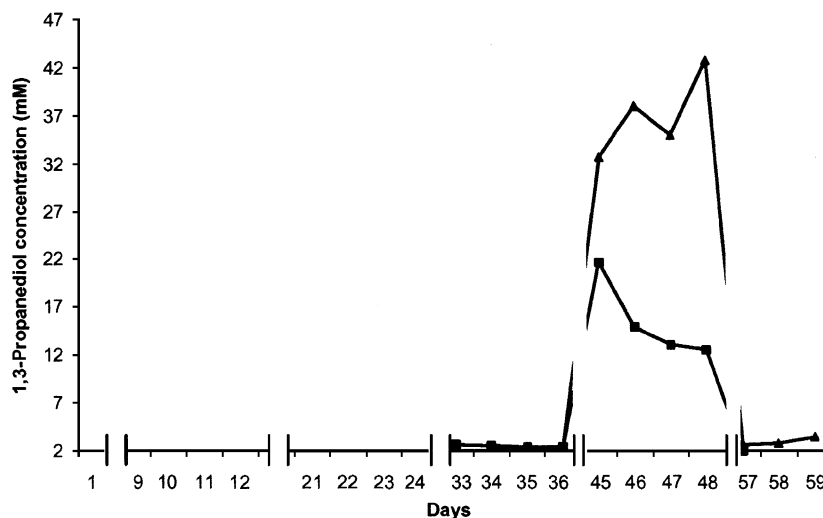

Fig. 3. Concentrations (mM) of 1,3-propanediol in reactor 1 and reactor 2: 1,3-propanediol (–▲–) in R1 (without *Lactobacillus reuteri* ATCC 55730); 1,3-propanediol (–■–) in R 2 (with *L. reuteri*). Data are means of duplicate analyses.

dramatic increase ($P < 0.05$) in 1,3-propanediol concentration to 37.0 ± 4.3 and 15.6 ± 4.2 mM for R1 and R2, respectively (Fig. 3). No reuterin accumulation was detected in fermentation samples, even during Lbr-100G.

Discussion

In this study, the effects of *L. reuteri* ATCC 55730 on adult intestinal microbiota and its capacity to secrete reuterin under conditions simulating the human proximal colon in the presence or absence of glycerol using an *in vitro* colonic model with immobilized fecal microbiota were investigated.

All bacterial populations tested in the fecal inoculum were retrieved in R1 and R2 at the end of Stab I. However, a new bacterial balance was established, with similar patterns in both reactors, which differed from that observed in the fecal inoculum. This type of change in population ratios is usually observed with *in vitro* intestinal fermentation systems (Macfarlane *et al.*, 1998; Cinquin *et al.*, 2006) and reflects changes due to applied fermentation conditions (retention time, culture medium, pH), which differ from conditions previously encountered in the host intestine. However, the different bacterial concentrations were within the range of physiological values for healthy adults as well as for other *in vitro* colonic fermentation models (Franks *et al.*, 1998; Probert & Gibson, 2004). Bacterial ecosystems established in R1 and R2 were therefore used to test *L. reuteri* ATCC 55730 and glycerol effects in the human colon.

The addition of glycerol in R1 (100G) strongly modified the SCFA ratio (by increasing butyrate) compared with Stab I. A marked increase in 1,3-propanediol production was also observed, which could explain the lack of effect of glycerol addition on SCFA concentration. Glycerol can be metabolized by various bacteria (Lin, 1976), but only a few enterobacteria (*Klebsiella pneumonia*, *Citrobacter freundii*, *Enterobacter agglomerans*), lactobacilli (*L. reuteri*, *Lactobacillus brevis*, *Lactobacillus collinoides* and *Lactobacillus buchneri*) and clostridia (*Clostridium butyricum*, *Clostridium perfringens* and *Clostridium pasteurianum*) are known to produce 1,3-propanediol from this polyol (Biebl *et al.*, 1999). Among these propanediol-producers, clostridia (especially *C. butyricum* and *C. perfringens*) are the most commonly reported intestinal species (Hill *et al.*, 1990), and contrary to *L. reuteri* and other propanediol-producers, they are the only ones known to produce butyrate as a by-product, together with 1,3-propanediol during glycerol fermentation (Biebl *et al.*, 1999). The metabolism of glycerol by commensal *C. butyricum* and/or *C. perfringens* might explain the simultaneous increase in 1,3-propanediol and butyrate during 100G. However, the bacterial intestinal ecosystem and the related metabolic pathways are very complex and many other commensal propanediol-producers, such as *L. reuteri*, *L. buchneri* and *L. brevis* as well as nonpropanediol producers, may be involved in the final metabolic effects of glycerol supplementation because the metabolism of intestinal bacteria is closely interrelated.

The introduction of *L. reuteri* ATCC 55730 in R2 (Lbr) changed the intestinal fermentation pattern compared with Stab I. The fermentative capacity of the microbiota increased due to a significant increase in propionic and acetic acids concentrations, while butyric acid decreased and lactic acid concentration remained low. These metabolic effects are consistent with the heterofermentative pattern of *L. reuteri*, which mainly produces lactic and acetic acids by the pentose phosphoketolase pathway. The absence of lactic acid accumulation in the medium was expected

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because lactic acid is an intermediate metabolite that is usually used by other intestinal bacteria.

The increase in lactobacilli–enterococci populations during 100G in R1 was not due to a stimulation of commensal *L. reuteri* because no increase was observed with the Lbre probe compared with Stab I. As expected, the addition of *L. reuteri* ATCC 55730 in R2 (Lbr) resulted in increased lactobacilli–enterococci population, but had no effects on *E. coli* concentration and on the other bacterial populations tested. However, a specific and significant decrease in *E. coli* was observed during Lbr-10G and Lbr-100G. This effect might be due to reuterin production from glycerol fermentation by *L. reuteri* ATCC 55730 although reuterin could not be detected by HPLC in the fermentation samples. *Escherichia coli* (pathogen and nonpathogen strains) are known to be very sensitive to reuterin (Axelsson *et al.*, 1989). The addition of glycerol alone (10G and 100G) or a small concentration of pure reuterin (1.3 mM) also induced a specific decrease in *E. coli* population. These data suggest the production of an inhibitory metabolite that specifically targets *E. coli* and has a similar activity to pure reuterin during intestinal fermentation in the presence of low (10 mM) and high (100 mM) glycerol concentrations, with or without *L. reuteri* ATCC 55370.

Reuterin is a highly reactive molecule due its aldehyde function that reacts with amino- or sulfhydryl groups in the medium or with bacteria (Casas & Dobrogosz, 2000). This reactivity might explain the lack of detection of reuterin, even during Lbr-100G. As for sugar fermentation, glycerol can also be converted to acetate, butyrate, lactate, succinate, ethanol, *n*-butanol and 2,3-butanediol and/or reduced to 1,3-propanediol via reuterin production. The accumulation of 1,3-propanediol, a typical product of glycerol fermentation not found in anaerobic conversions of other organic substrates (Biebl *et al.*, 1999), showed that glycerol was metabolized via the reuterin pathway. Although it is believed that some strains of *L. reuteri* are unique among propanediol producers in their ability to accumulate reuterin from glycerol (Casas & Dobrogosz, 2000), few enterobacteria and *C. butyricum* can accumulate reuterin during *in vitro* fermentation of glycerol (Barbirato *et al.*, 1996; Gonzalez-Pajuelo *et al.*, 2005). These data suggest that small amounts of reuterin were produced in R1 during addition of glycerol by intestinal propanediol-producers such as *C. butyricum* or commensal *L. reuteri*, because small concentrations of *L. reuteri* were detected in fermentation samples from R1. The lower 1,3-propanediol concentration measured during Lbr-100G in R2 compared with 100G in R1 might be explained by the fact that *L. reuteri* ATCC 55730 is much more effective for producing and accumulating reuterin compared with other propanediol producers, which mostly produce reuterin as a transient metabolite that is immediately reduced to 1,3-propanediol (Casas & Dobrogosz, 2000; Vollenweider & Lacroix, 2004). In this case, reuterin formed by *L. reuteri* ATCC 55730 could rapidly react with amino or sulfhydryl groups, escaping from the conversion to 1,3-propanediol. The *E. coli* population decreased similarly by 0.4–0.6 log cells mL^{-1} with the addition of pure reuterin (1.3 mM), or glycerol (10G and 100G) in the presence or absence of *L. reuteri* ATCC 55730.

These data strongly suggest that the decrease in *E. coli* concentrations observed during addition of glycerol (in presence or absence of *L. reuteri* ATCC 55730) was due to *in situ* reuterin production. A very low reuterin concentration, and low glycerol concentration were sufficient to induce a significant reduction in *E. coli* population whereas the combination of *L. reuteri* ATCC 55730 with glycerol did not improve the effect. The key question is now to determine how much glycerol (either derived directly from ingestion, produced by bacterial lipase or fermentation of desquamed epithelial cells) is readily available *in vivo* for bacterial conversion within the human intestine. Further experiments with more sensitive methods (for example with ^{14}C-enriched glycerol) should also be performed *in vitro*, as well as *in vivo* with animal studies simulating different human populations (young infants or patients with short-bowel syndrome) to follow the intestinal metabolism of glycerol and investigate its effects on bacterial populations, activity and host health.

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