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Comparative effects of exopolysaccharides from lactic acid bacteria and fructo-oligosaccharides on infant gut microbiota tested in an *in vitro* colonic model with immobilized cells

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three-stage *in vitro* colon model; exopolysaccharides; fructo-oligosaccharides; cell immobilization; infant; microbiota.

Abstract

The aim of this study was to compare the effects of purified exopolysaccharides from *Lactobacillus rhamnosus* RW-9595M with those of a well-known prebiotic (short-chain fructo-oligosaccharides) on infant colonic microbiota using a new three-stage chemostat model with immobilized infant faecal microbiota. Two continuous cultures with different faecal inocula were tested with different compositions of carbohydrate media. During the first fermentation (F1), fructo-oligosaccharides tested at a concentration of 9.8 g L^{-1} increased the number of lactobacilli and decreased coliforms both in gel beads and in effluent from all three reactors, in agreement with data from the literature. During the second fermentation (F2), the effect of fructo-oligosaccharides tested at a lower concentration (7.5 g L^{-1}) was reduced compared with F1. Fructo-oligosaccharides also increased total organic acid concentration and decreased ammonia production. Results obtained for exopolysaccharide tested at 1.5 g L^{-1} indicate that exopolysaccharides from *L. rhamnosus* RW-9595M was not metabolized by infant microbiota and lacked any prebiotic effect.

Introduction

The colonic microbiota is a complex ecosystem having great effects on human health. Such complexity involves host requirements for bacterial fermentation products, colonization resistance to microbial pathogens, activation or destruction of genotoxins and mutagens, and modulation of immune system functions (Macfarlane & McBain, 1999). Throughout life, a succession of bacterial equilibria occurs in the intestinal human microbiota. It is during infancy, however, that the bacterial composition and activity of the intestinal ecosystem are established, factors that contribute greatly to the foundation of health. Indeed, it has been suggested that interactions between intestinal microbes and host not only have short-term effects on immune functions and early development but could also exert long-term effects on health, disease and mortality risks in adulthood (Reid, 2004).

In infancy, the microbial ecology of the gut is strongly influenced by diet. It is generally believed that breast-fed infants have a microbiota dominated by bifidobacteria and lactobacilli, creating a protective environment against pathogens. Formula-fed infants, having a more complex microbiota, are more susceptible to intestinal disorders (Mountzouris *et al.*, 2002). Human milk contains a high amount of oligosaccharides, which stimulate the growth of indigenous bifidobacteria and lactobacilli and may contribute to the protection of breast-fed infants against infections and inflammations (Kunz & Rudloff, 1993).

Attempts have been made to modulate the microbial balance of colonic microbiota with the aim of improving the health and well-being of formula-fed infants. This can be achieved either by oral administration of live microorganisms known as probiotics, or by consumption of nondigestible food ingredients that selectively stimulate the growth and/or activity of beneficial bacteria (Bakker-Zierikzee *et al.*, 2005). The main bacterial populations targeted by these food ingredients, also called prebiotics, are bifidobacteria and lactobacilli, which are known to have health-promoting properties such as inhibition of exogenous pathogens.

The most effective prebiotics in infant diet are oligosaccharides such as fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) (Ghisolfi, 2003). The most studied prebiotics, FOS, escape the upper gastrointestinal (GI)

digestion, and once in the colon exert a bifidogenic effect causing decreases in *Bacteroides* spp. and clostridia counts. FOS also promote an increase in short-chain fatty acids (SCFA) concentration, particularly of butyrate (Gibson *et al.*, 1995; Rastall & Gibson, 2002). It has been suggested that other food compounds such as exopolysaccharides (EPS) from lactic acid bacteria exert prebiotic effects on human intestinal microbiota (Dal Bello *et al.*, 2001), although only data from *in vitro* tests have been published. EPS are produced by numerous bacteria in association with their cell surface or are excreted in the fermentation medium. They are believed to be implicated in bacterial protection (Roberts, 1996), and are widely used in the food industry to improve food texture because of their rheological properties (De Vuyst & Degeest, 1999). In the past decade, the interest in EPS produced by lactic acid bacteria has grown because of their GRAS (generally regarded as safe) status (Sutherland, 1998) and their putative health-promoting effects (Ruas-Madiedo *et al.*, 2002).

In this study, we tested the effects of an EPS from *L. rhamnosus* RW-9595M and FOS on infant microbial ecology using a new three-stage continuous fermentation colonic model with immobilized faecal microbiota (Cinquin *et al.*, 2004, 2006). Composition and activity of infant microbiota as affected by carbohydrate composition were monitored during two continuous culture experiments carried out over 33 and 34 days. The effects of EPS and FOS were also specifically tested on four bifidobacterial species commonly encountered in infant faeces using a set of bifidobacterial species-specific 16S rRNA-targeted oligonucleotide probes and fluorescence *in situ* hybridization (FISH).

Materials and methods

Bacterial strains

Lactobacillus rhamnosus RW-9595 M, a high EPS-producer strain, was obtained from the Lactic Acid Bacteria Research Network (RBL Network) culture collection (Dairy Research Centre STELA, Université Laval, Québec, QC). This culture was isolated from *L. rhamnosus* ATCC 9595M and shown to be a stable EPS mutant (Dupont *et al.*, 2000). EPS from *L. rhamnosus* RW-9595M are large polymers with high molecular weight (5×10^7 Da) and a repeated unit composed of pyruvate, 1; D-glucose, 2; D-galactose, 1; and L-rhamnose, 4 (Van Calsteren *et al.*, 2002).

Bacterial strains used for probe validation and listed in Table 1 were obtained from the Deutsche Sammlung von Milkoorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). They were cultivated in de Man Rogosa and Sharp (MRS) broth supplemented with cysteine (0.05%) under anaerobic conditions ($CO_2 : N_2$) at 37 °C.

EPS production and purification

EPS production and quantification were carried out as described by Bergmaier *et al.* (2001). Briefly, the strain was subcultured twice in MRS broth medium before being inoculated at 10% (volume in volume, v/v) in basal minimum medium (BMM) (Morishita *et al.*, 1981) supplemented with glucose ($40\,g\,L^{-1}$) and tryptone ($2\,g\,L^{-1}$) in a 15 L working-volume bioreactor (Inox-tech Inc., Montreal, QC). Fermentation was carried out aerobically at 37 °C and controlled at pH 6 by the addition of 6 N NH_4OH for 48 h. The fermented medium was then heated at 100 °C for 15 min in order to inactivate bacteria and to denature EPS-degrading enzymes, cooled and centrifuged (25 min, 13 200 ***g***) to remove bacteria. The supernatant (15 L) was ultrafiltrated and diafiltrated with four volumes of water using a 50 kDa molecular weight cut-off membrane (Romicon, Worburn, MA) to reach a final volume of 2 L, and then freeze-dried. Two EPS powders were prepared, one for F1 and one for F2. EPS in the powder was quantified with a phenol-sulphuric test (Dubois *et al.*, 1956), residual low-molecular-weight carbohydrates were tested in the final filtrate and reported to powder composition, and protein content was evaluated with the Lowry test.

Faecal sample collection, preparation and immobilization

Faecal samples used for immobilization were collected from two healthy 6-month-old infants and prepared for immobilization as previously described (Cinquin *et al.*, 2006). The immobilization procedure was based on a dispersion process in a two-phase system, as described (Cinquin *et al.*, 2004). The autoclaved polymer solution [gellan gum 2.5% (weight in volume, w/v), xanthan gum 0.25% (w/v), sodium citrate 0.2% (w/v)] was cooled to 43 °C and inoculated aseptically with the faecal inoculum (2%, v/v). At the end of the immobilization procedure, beads with diameters in the range 1.0–2.0 mm were selected by wet-sieving and used for fermentation. The entire process was completed under aseptic conditions within 1 h.

Fermentation procedures

The continuous culture fermentation system used in this study was described by Cinquin *et al.* (2006). Briefly, this system consisted of three custom-stirred flat-bottom glass bioreactors (R1, R2 and R3) arranged in series with working volumes of 100, 125 and 100 mL, respectively, and used to simulate pH and retention times encountered in the proximal (PCS), transverse (TCS), and distal (DCS) parts of an infant colon. The first reactor was inoculated with 30% (v/v) of freshly prepared beads. Retention times were set at 4 h (R1), 5 h (R2) and 4 h (R3) by adjusting the feed flow rate to

Table 1. Bacterial strains used for the fluorescence *in situ* hybridization probe specificity tests with conditions described in Table 2

	Strains	Blong209	Bbr*	PAD†	pBiBIF‡	Bif164§
Bifidobacteria						
B. longum bv. *infantis*¶	DSMZ 20088	+++	–	–	–	+++
B. longum bv. *suis*	DSMZ 20211	++	–	–	–	+++
B. longum bv. *longum*¶	DSMZ 20219	+++	–	–	–	+++
B. breve¶	DSMZ 20213	–	+++	–	–	+++
B. adolescentis¶	DSMZ 20083	–	–	+++	–	+++
B. bifidum¶	DSMZ 20456	–	–	+++	+++	–
B. cuniculi	DSMZ 20435	–	–	–	+++	+++
B. pseudolongum ssp. *pseudolgum*	DSMZ 20099	–	–	–	–	+++
B. animalis ssp. *animalis*	DSMZ 20104	–	–	–	–	++
B. indicum	DSMZ 20214	–	–	–	–	+
B. animalis ssp. *lactis*	DSMZ 10140	–	–	–	–	+++
B. saeculare	DSMZ 6531	–	–	–	–	++
B. subtile	DSMZ 20096	–	–	–	–	+++
B. pullorum	DSMZ 20433	–	–	–	–	+++
B. dentium	DSMZ 20436	–	–	–	–	+++
B. pseudocatenulatum¶	DSMZ 20438	++	–	–	–	+++
B. boum	DSMZ 20432	–	–	–	–	+++
B. thermophilum¶	DSMZ 20210	–	–	–	–	+++
B. angulatum¶	DSMZ 20098	–	–	–	–	+++
B. merycicum	DSMZ 6492	–	–	–	–	+++
B. catenulatum¶	DSMZ 20224	+++	–	–	–	+++
	Strains	Blong209	Bbr	PAD	pBiBIF	EUB 338‖
Intestinal bacteria						
Collinsella aerofaciens	DSMZ 3979	–	–	–	–	+++
Eubacterium eligens	DSMZ 3376	–	–	–	–	++
Eubacterium biforme	DSMZ 3989	–	–	–	–	+++
Ruminococcus productus	DSMZ 2950	–	–	–	–	+++
Bacteroides fragilis	DSMZ 2151	–	–	–	–	+++
Enterococcus faecium	DSMZ 20477	–	–	–	–	+++
Streptococcus salivarius	DSMZ 20560	–	–	–	–	+++
Lactobacillus fermentum	Our collection	–	–	–	–	++
Lactobacillus acidophilus	DSMZ 20079	–	–	–	–	+++

*Germond *et al*. (2002).
†Yamamoto *et al*. (1992).
‡Matsuki *et al*. (2004).
§Langendijk *et al*. (1995).
¶Bifibobacteria species occurring in faecal samples.
‖Amann *et al*. (1995).
+++ and ++: strong and medium fluorescent signal, respectively.

25 mL h^{-1}. The pH was maintained at 5.9 and 6.2 in R1 and R2, respectively, whereas pH in R3 (DCS) was monitored but not controlled because it stabilized at physiological levels (6.5–6.6).

For both fermentation experiments, effluent samples (4 mL) were collected daily in the three reactors. Bacteriological analyses with plate counts were performed daily. Metabolite and FISH analyses were performed for the last 3 and 2 days of each fermentation period, respectively. Bead samples were collected just after the immobilization process, after 48 h of batch cultures used for bead colonization, and during the last day of the pseudo-steady-state periods, for plate counts and microscopy analyses. The pseudo-steady state was reached when bacterial populations in fermentation effluents from all reactors did not change by more than 0.5 Log10 units, this corresponding to the last 3 days for each condition.

Fermentation media

Two independent fermentations were carried out to compare the prebiotic effects of FOS containing 95% of oligofructose with an average degree of polymerization (DP) of 3.6 monomers (Nutraflora® P-95 Canacure, Laval, QC)

with those of EPS produced by *L. rhamnosus* RW-9595M. Fermentation media simulating an infant ileal chyme were based on the composition described by Cinquin *et al.* (2004, 2006). The medium composition was calculated from the composition of a standard infant formula supplemented with rice starch (Sigma, St Louis, MO) and applying digestibility indices from the literature. FOS tested at 7.8 and 9.5 g L^{-1} medium corresponds to a diet containing between *ca*. 3.5% and 7.5% FOS among carbohydrates, for a digestibility index of 11% (Molis *et al*., 1996). The ratio of the different carbohydrates in the two control media with maltodextrin (10 and 13 g L^{-1} total carbohydrate for M-10 and M-13, respectively) remained unchanged, with 7% starch, 18% lactose and 75% maltodextrin in both experiments (Table 3).

During the first fermentation (F1), which lasted for 34 days, the first two substrates tested were FOS (FOS-13) and maltodextrin (M-13) at 9.8 g L^{-1}, i.e. 75% of the total carbohydrate concentration (13 g L^{-1}) (Table 3). During the third and fourth periods, it was not possible to substitute EPS for maltodextrin because of high medium viscosity, which limited EPS addition into the medium to a maximum level of 1.5 g L^{-1}. Therefore, for the EPS test, the total carbohydrate concentration was decreased to 10 g L^{-1} in order to reach a significant EPS percentage of 15% (EPS-10) in the medium carbohydrate fraction. During the third period (M-10), maltodextrin was reduced to 7.5 g L^{-1} (75% of the total carbohydrate concentration in the medium with 10 g L^{-1}) in order to use this treatment as a control for EPS-10 (Table 3). The second fermentation (F2), which lasted for 32 days, was performed to assess whether the effects observed during the fourth period of F1 (EPS-10) were the result of the addition of EPS or of a lack of assimilable carbohydrates, since EPS substituted for equivalent amounts of maltodextrin in the medium. EPS (1.5 g L^{-1}) was then added to medium M-10 containing 10 g L^{-1} carbohydrates (EPS-11.5). This medium was compared with two other media containing either 10 (M-10) or 11.5 (M-11.5) g L^{-1} total carbohydrates, with maltodextrin. A medium with a low FOS concentration (7.5 g L^{-1} substituting for an equivalent amount of maltodextrin) and 10 g L^{-1} total carbohydrates was also tested (FOS-10).

Bacterial enumeration

Plate counts were performed within 1 h of sampling. Samples were serially diluted 10-fold with peptone water (0.1%, pH 7.0). Four 20 μL drops of each dilution were plated in duplicate on both selective and nonselective media, as described previously (Cinquin *et al*., 2004).

Fluorescence *in situ* hybridization analyses were performed for the detection of bifidobacteria and total bacterial populations (Table 2) in fermentation samples from DCS (last 2 days of the pseudo-steady state of each fermentation period). Samples were fixed with paraformaldehyde (PFA) 4%, dehydrated with ethanol baths, and applied to glass slides as described previously (Cinquin *et al*., 2006). The slides were hybridized with the various probes (see next section) and stained with 4′,6-diamidino-2-phenylindole (DAPI). Cells were then counted visually with an Olympus BX 51 epifluorescence microscope (Olympus America, Melville, NY) equipped with a monochrome camera, as already described (Cinquin *et al*., 2006). Each assay was done in duplicate.

Probe design and specificity for bifidobacteria

Four bifidobacterial species-specific 16S rRNA-targeted oligonucleotide probes were used in this study (Table 2). The Bbr probe was previously designed to be used as a forward PCR primer for *Bifidobacterium breve* (Germond *et al*., 2002). The pBiBIF and PAD probes were used in dot blot and FISH for the detection of *Bifidobacterium bifidum* (Matsuki *et al*., 2004) and *Bifidobacterium adolescentis* (Yamamoto *et al*., 1992), respectively. An oligonucleotide probe targetting the small subunit rRNA sequence of *Bifidobacterium longum* (including *B. suis*, *B. infantis* and *B. longum*) (Sakata *et al*., 2002) was designed with the Arb software package (Ludwig *et al*., 2004), the Check-Probe function of the RDP software package (Maidak *et al*., 1997), and the EMBL database. This probe was named (S-S-Blong-

Table 2. Sequences of oligonucleotide probes used for fluorescence *in situ* hybridization analyses of faecal and fermentation samples and optimal conditions for hybridization specificity

			Temperature (°C)		
Sequence (5′–3′)	Targeted	Formamide (%)	T_H	T_W	References
Eub338 5′-GCT GCC TCC CGT AGG AGT-3′	Universal eubacterial probe	0	46	48	Amann *et al*. (1995)
Bif164 5′-CAT CCG GCA TTA CCA CCC-3′	Bifidobacteria	0	46	48	Langendijk *et al*. (1995)
PAD 5′-GCT CCC AGT CAA AAG CG-3′	*Bifidobacterium adolescentis*	15	50	52	Yamamoto *et al*. (1992)
Bbr 5′-GCT CGC ACT GTC GCA TC-3′	*Bifidobacterium breve*	0	50	52	Germond *et al*. (2002)
Blong209 5′-CCC ATC CCA TAC CGC GAA-3′	*Bifidobacterium longum*	0	54	56	This study
pBiBIF 5′-CCA CAA TCA CAT GCG ATC ATG-3′	*Bifidobacterium bifidum*	0	50	52	Matsuki *et al*. (2004)

T_H, hybridization temperature; T_W, washing temperature.

0209-b-A-18) according to the nomenclature suggested by the Oligonucleotide Probe Database (OPD) (Alm *et al.*, 1996). In this paper, the probe name is abbreviated Blong209.

Hybridization specificity for FISH was optimized for each probe by testing several temperatures and formamide concentrations (up to 35%) in the hybridization buffer to increase the stringency of the annealing reaction. Bif164 and Eub338 probes were used as positive controls for bifidobacteria strains and intestinal bacteria, respectively. Probe validation was performed with 21 bifidobacterial species as well as with nine other bacterial species commonly encountered in human faeces (Table 1).

Metabolites analyses

Short-chain fatty acids (SCFA) and isoacids production was analysed by high-performance liquid chromatography (HPLC), and ammonia concentration with an ammonia electrode, as described by Cinquin *et al.* (2006). Each assay was done in duplicate. Mean metabolite concentrations were calculated for the last 3 days of fermentation; SCFA and isoacids were expressed in mmol per L (mM), and ammonia concentration was expressed in mmol per kg of wet-weight faeces or in mM for fermentation samples.

Quantification of residual EPS in fermentation effluent samples from DCS

Quantification of residual EPS was performed with fermentation samples from DCS for the last 2 days of EPS periods (EPS-10 and EPS-11.5). Samples were heated at 100 °C for 15 min, cooled and centrifuged (25 min, 13 200 ***g***) to remove bacteria. An enzymatic treatment was applied to the supernatant to hydrolyze residual starch and glycoprotein (mucin), which can interfere with the EPS test. Alpha-amylase (150 μL) (Sigma-Aldrich, St Louis, MO) was added to the supernatant (2 mL) and incubated for 1 h at room temperature. Pronase E (100 μL) (Sigma-Aldrich) was then added and incubated for 20 h at 37 °C. After the enzymatic treatment, samples were ultrafiltrated and diafiltrated to eliminate residual low-molecular-weight sugars in the filtrate and recover nonmetabolized EPS using the method developed by Bergmaier *et al.* (2001). EPS in sample concentrates was quantified using the phenol-sulphuric test (Dubois *et al.*, 1956). Ultrafiltrations were performed in duplicate for each sample.

Statistical analyses

A one-way analysis of variance (ANOVA) was performed using JMPIN® (SAS Institute Inc., Cary, NC) to test the effects of FOS and EPS on bacterial populations and metabolite production during the last 3 days of the fermentation periods. When significant differences were found below a probability level of 0.05, treatment means were compared using the Tukey–Kramer-HSD test.

RESULTS

Probe design and evaluation of probe specificity

Four species-specific probes were used for FISH analyses of faecal and fermentation samples (Table 2). Blong209 was designed in this study to detect *Bifidobacterium longum* (including the former *Bifidobacterium infantis*, *Bifidobacterium suis* and *B. longum*) (Sakata *et al.*, 2002). The target region used to design the oligonucleotide probe specific for *B. longum* was fully complementary to 16S rRNA of *B. infantis, B. longum* and *B. suis,* but also to *Bifidobacterium indicum* and *Bifidobacterium pseudolongum* (Fig. 1). The specificity of the four probes used in this study was tested against 21 bifidobacterial species and nine other bacterial species commonly encountered in human faeces (Table 1). For each probe a good fluorescent signal was obtained. Under our FISH conditions (Table 2), the PAD and Bbr probes specifically hybridized with *Bifidobacterium adolescentis* and *Bifidobacterium breve*, respectively. pBiBIF hybridized with both *Bifidobacterium bifidum* and *Bifidobacterium cuniculi*, and Blong209 hybridized with its target species *B. longum*, but also with *Bifidobacterium pseudocatenulatum* and *Bifidobacterium catenulatum*, with only three mismatchs at the 5′ end of the 16S rRNA target region

Blong209	3' AAG CGC CAT ACC CTA CCC 5'
Target	5' UUC GCG GUA UGG GAU GGG 3'
Bifidobacterium longum bv *infantis* (DSMZ 20088)	=== === === === === ===
Bifidobacterium indicum (DSMZ 20214)	=== === === === === ===
Bifidobacterium longum bv *longum* (DSMZ 20219)	=== === === === === ===
Bifidobacterium pseudolongum (DSMZ 20099)	=== === === === === ===
Bifidobacterium longum bv *suis* (DSMZ 20211)	=== === === === === ===
Bifidobacterium pseudocatenulatum	=Ca U== === === === ===
Bifidobacterium merycicum (DSMZ 6492)	=Ca U== === === === ===
Bifidobacterium subtile (DSMZ 20096)	==a U== =C= === === ===
Bifidobacterium adolescentis (DSMZ 20083)	==a U== === === === ===
Bifidobacterium catenulatum (ATCC 27539)	=Ca U== === === === ===

Fig. 1. Sequence of the Blong209 probe shown on the top of a 16S rRNA gene alignment (region 209–226 according to *Eshcerichia coli* consensus numbering) for *Bifidobacterium longum* and related species (bv : biovar). Dashes indicate full complementarity with the target sequence. There are at least three mismatches with sequences of nontarget microorganisms.

(Fig. 1). Surprisingly, Blong209 did not hybridize with *B. pseudolongum*, nor with *B. indicum*.

Effect of FOS and EPS on immobilized cell populations

All bacterial populations detected in faecal samples were also present in beads for both fermentations. At the end of the first stabilization period (M-13 and M-10 for F1 and F2, respectively), the differences in bacterial counts of beads between F1 and F2, and between beads and their respective inocula were generally below 0.7 Log10 CFU g^{-1}. The exceptions were for staphylococci, lactobacilli, Gram-positive cocci and clostridia in F1, and for bifidobacteria and clostridia in F2, which showed greater differences between faecal inoculum and beads, and, for staphylococci, lactobacilli and clostridia, between the two faecal inocula.

During F1, the replacement of 9.5 g L^{-1} of maltodextrin (M-13) in the medium by FOS (FOS-13) induced an increase in bifidobacteria (from 9 to 9.8 Log10 CFU mL^{-1}) and lactobacilli (from 5.3 to 6.3 Log10 CFU mL^{-1}), and a decrease in coliforms (from 9.6 to 7.3 Log10 CFU mL^{-1}). The reduction in total carbohydrate concentration of the medium from 13 (for M-13 and FOS-13) to 10 g L^{-1} (M-10) induced a general decrease (minus 0.4 to 2.5 Log10) for most populations in beads, except for coliforms and clostridia, which increased by 1.2 and 0.8 Log10 CFU mL^{-1}, respectively, compared with FOS-13. The replacement of 1.5 g L^{-1} maltodextrin (M-10) by EPS (EPS-10) led to a decrease in clostridia counts (from 7.3 to 6.1 Log10 CFU mL^{-1}), and an increase for staphylococci (from 6.9 to 8.6 Log10 CFU mL^{-1}).

For F2, only limited changes in bacterial counts occurred during supplementation of M-10 with 1.5 g L^{-1} of EPS (EPS-11.5). Compared with M-10, FOS-10 resulted in decreases of staphylococci (from 7.7 to 5.9 Log10 CFU mL^{-1}) and coliforms (from 9.7 to 7.3 Log10 CFU mL^{-1}). The increase in total carbohydrates from 10 (M-10) to 11.5 (M-11.5) g L^{-1} induced a decrease in bacteroides (from 9.2 to 8.3 Log10 CFU mL^{-1}), staphylococci (from 7.7 to 5.9 Log10 CFU mL^{-1}), coliforms (from 9.7 to 6.4 Log10 CFU mL^{-1}) and clostridia (from 8.5 to 7.5 Log10 CFU mL^{-1}) counts.

Effects of FOS and EPS on free cell populations

Counts for the main bacterial populations in the effluents from the three reactors and two fermentations were affected by the carbohydrate composition of the medium (Table 4). The replacement of maltodextrin (M-13) by FOS (FOS-13) in F1 induced an increase in bifidobacteria and lactobacilli numbers in R3 (DCS), whereas bacteroides, coliforms, and clostridia decreased ($P < 0.05$). This effect was also significant in R1 and R2 for lactobacilli and in R2 for coliforms. When the system was fed with M-10 during the third period, bacterial populations in the effluent from R3 were very close to those for M-13, except for coliforms, which were lower. Compared with M-10, replacement of 1.5 g L^{-1} maltodextrin by EPS (EPS-10) induced a significant increase in facultative anaerobes ($P < 0.0005$) in all reactors, mainly owing to an increase in staphylococci counts, and in Gram-positive cocci to a lesser extent. A significant decrease of lactobacilli was also observed in R1 and R3 for EPS-10 compared with M-10.

Table 3. Carbohydrate composition of fermentation media tested during two continuous colonic fermentation experiments

Fermentation 1	M-13	FOS-13	M-10	EPS-10
Time period (days)	2–14	15–22	23–29	30–34
Total carbohydrates (g L^{-1})	13	13	10	10
Maltodextrin (g L^{-1})	9.8	–	7.5	6
FOS (g L^{-1})	–	9.8	–	–
EPS (g L^{-1})	–	–	–	1.5
Lactose (g L^{-1})	2.3	2.3	1.8	1.8
Rice starch (g L^{-1})	0.9	0.9	0.7	0.7
Fermentation 2	M-10	EPS-11.5	FOS-10	M-11.5
Time period (days)	2–11	12–18	19–25	26–33
Total carbohydrates (g L^{-1})	10	11.5	10	11.5
Maltodextrin (g L^{-1})	7.5	7.5	–	9
FOS (g L^{-1})	–	–	7.5	–
EPS (g L^{-1})	–	1.5	–	–
Lactose (g L^{-1})	1.8	1.8	1.8	1.8
Rice starch (g L^{-1})	0.7	0.7	0.7	0.7

During the second fermentation experiment (F2), the addition of 1.5 g L^{-1} of EPS (EPS-11.5) to medium M-10 with maltodextrin induced a significant decrease in staphylococci, Gram-positive cocci and lactobacilli, with major effects observed in R3 (DCS), accompanied by a decrease in total anaerobic populations. On the other hand, EPS-11.5 induced significantly higher numbers for Gram-positive cocci (R1 and R3) and staphylococci (all three reactors), and lower counts for lactobacilli (all reactors) compared with M-11.5. The replacement of 7.5 g L^{-1} maltodextrin (M-10) by FOS (FOS-10) did not result in increases of bifidobacteria nor of lactobacilli populations, but lower numbers of Gram-positive cocci and staphylococci were observed in all three reactors. However, the switch from FOS-10 to M-11.5 did not change bacterial populations in the effluents, except for staphylococci, which decreased significantly in R1 and R3, and lactobacilli, which slightly increased in all three reactors.

Effect of FOS and EPS on bifidobacterial species in effluent fermentation

Probes Eub338 and Bif164 were used to measure total anaerobic populations and bifidobacteria. Four species-specific probes for *B. longum*, *B. breve*, *B. adolescentis* and

Table 4. The effect of medium carbohydrate composition on bacterial populations measured by plate counts in effluents from the three reactors for two fermentation experiments

	Fermentation 1[ab]				Fermentation 2[ab]			
	M-13	FOS-13	M-10	EPS-10	M-10	EPS-11.5	FOS-10	M-11.5
PCS (R1)								
Facultative anaerobes	7.9[a]	7.9[a]	8.0[a]	8.7[b]	8.2[a]	7.9[a]	8.0[a]	8.0[a]
Total anaerobes	10.0[a]	10.1[a]	9.8[a]	10.0[a]	9.9[a]	10.1[a]	10.1[a]	10.0[a]
Bifidobacteria	9.6[a]	9.9[a]	9.4[ab]	8.9[b]	9.7[a]	9.8[a]	9.5[a]	9.7[a]
Bacteroides	7.9[a]	7.6[a]	7.9[a]	8.0[a]	7.9[a]	8.0[a]	7.6[b]	7.8[ab]
Gram + cocci	7.9[a]	7.4[a]	7.7[a]	8.4[b]	8.0[a]	7.7[a]	7.3[b]	7.0[b]
Staphylococci	7.5[ab]	7.2[a]	7.8[b]	8.3[c]	6.7[a]	5.7[b]	6.1[b]	4.3[c]
Coliforms	7.8[a]	7.6[ab]	6.8[b]	7.3[ab]	7.6[a]	7.8[a]	7.6[a]	8.0[a]
Lactobacilli	5.2[a]	6.4[b]	5.6c	4.9[d]	6.3[ab]	5.9[c]	6.1[bc]	6.5[a]
Clostridia	7.5[a]	7.2[a]	7.7[a]	7.1[a]	6.6[a]	6.6[a]	6.5[a]	6.5[a]
TCS (R2)								
Facultative anaerobes	8.2[a]	8.2[a]	8.5[b]	8.7[c]	8.3[a]	8.0[a]	8.1[a]	8.0[a]
Total anaerobes	10.3[a]	10.1[a]	10.1[a]	10.1[a]	10.0[a]	10.0[a]	10.0[a]	10.1[a]
Bifidobacteria	9.6[a]	9.9[a]	9.8[a]	9.8[a]	9.4[a]	9.5[a]	9.6[a]	9.7[a]
Bacteroides	8.3[ab]	8.0[a]	8.8[b]	8.7[b]	8.8[a]	8.7[a]	8.2[b]	8.2[b]
Gram + cocci	8.1[a]	8.1[a]	8.3[ab]	8.4[b]	7.9[a]	7.7[a]	7.3[b]	7.1[b]
Staphylococci	7.9[a]	7.9[a]	7.9[a]	8.4[b]	6.4[a]	5.7[b]	5.4[bc]	4.8[c]
Coliforms	8.1[a]	7.5[b]	7.3[b]	7.4[b]	8.0[a]	8.0[a]	8.1[a]	7.9[a]
Lactobacilli	5.7[a]	6.5[b]	5.9[a]	5.8[a]	6.5[ac]	5.8[b]	6.1[a]	6.5[c]
Clostridia	7.5[a]	7.5[a]	7.5[a]	7.5[a]	6.8[a]	6.6[a]	6.5[a]	6.8[a]
DCS (R3)								
Facultative anaerobes	7.9[a]	7.7[a]	8.0[a]	8.7[b]	8.2[a]	8.1[a]	8.2[a]	8.0[a]
Total anaerobes	9.9[a]	9.9[a]	9.8[a]	9.9[a]	10.0[a]	9.6[b]	9.9[a]	9.9[a]
Bifidobacteria	9.2[a]	9.8[b]	9.3[a]	8.8[c]	9.4[a]	9.1[a]	9.1[a]	9.2[a]
Bacteroides	7.8[a]	7.5[b]	8.1[ac]	8.3[c]	8.8[a]	8.6[ab]	8.4[b]	8.5[b]
Gram + cocci	7.9[ab]	7.1[b]	7.8[ab]	8.3[b]	8.0[a]	7.4[b]	7.1[b]	7.1[b]
Staphylococci	6.0[a]	5.8[a]	6.7[ab]	7.5[b]	6.5[a]	5.6[b]	5.4[b]	4.5[c]
Coliforms	7.9[a]	7.2[b]	7.0[b]	7.1[b]	8.1[a]	8.0[a]	7.9[a]	7.9[a]
Lactobacilli	5.4[ac]	6.3[b]	5.8[a]	4.9[c]	6.6[a]	5.8[b]	6.1[b c]	6.4[c]
Clostridia	7.4[a]	6.8[b]	7.6[a]	7.3[a]	6.8[a]	6.4[b]	6.0[c]	5.7[c]

PCS, proximal colon simulation; TCS, transverse colon simulation; DCS, distal colon simulation.

[a]Mean values for the three last days of pseudo-steady-state periods, expressed in Log10 (CFU per mL).

[b]Effect of carbohydrate substrates on bacterial populations: values with different letters in a row are significantly different with the Tukey–Kramer HSD test ($P < 0.05$).

B. bifidum were used to monitor bifidobacteria counts in faeces and fermentation samples from R3 for the two fermentation experiments (Table 2). Faeces from baby 2 used in F2 were highly colonized by bifidobacteria, which represented 90% of the anaerobic populations measured with Eub338 (Table 5). The total bifidobacterial population from baby 2 was dominated by *B. breve* (10.5 Log10 CFU mL^{-1}), followed by *B. longum* (10.1 Log10 CFU mL^{-1}); *B. adolescentis* and *B. bifidum* were not detected. All four bifidobacterial species were detected in F2, whereas *B. adolescentis* and *B. bifidum* were not detected in F1 (Table 5). In both fermentations, *B. longum* was the dominant species, accounting for 43–76% of the total bifidobacterial population measured with Bif164.

During F1, FOS-13 treatment did not change the total bifidobacterial population tested with Bif164 compared with M-13, which was not different from M-10. However, bifidobacteria counts were lower during EPS-10 compared with M-10, although this difference was not statistically significant. No statistical difference was detected for *B. longum* and *B. breve* among tested treatments.

In F2, total bifidobacteria numbers did not change with medium composition, as for *B. breve*, *B. adolescentis*, and *B. bifidum* counts. However, FOS-10 led to a significant increase in *B. longum* counts compared with M-10 and EPS-11.5. No significant effect was observed when FOS-10 was compared with M-11.5, which was not significantly different from M-10 and EPS-11.5. The proportion of *B. longum* in the total bifidobacteria populations (76%) was higher than in the other treatments (43–67%).

Effects of FOS and EPS on metabolic activities

Total SCFA concentrations ranged from 97.1 to 197.4 mM and generally increased from R1 to R3 for both F1 and F2

Table 5. The effect of medium carbohydrate composition on bifidobacteria populations determined with fluorescence *in situ* hybridization (FISH) analyses in effluent samples from distal colon simulation (R3) during two colonic fermentations F1 and F2

	Fermentation F1[ab]									
	M-13		FOS-13		M-10		EPS-10		Faeces	
	FISH counts	%	FISH counts	%	FISH counts	%	FISH counts	%	FISH counts	%
Eub338	10.6		10.5		10.5		10.2			
Bif164	10.4[a]	71[c]	10.4[a]	84[b]	10.3[ab]	76[b]	10.1[b]	80[b]	Nd	
Blong209	10.3[a]	74	10.3[a]	66	10.1[a]	57	9.9[a]	61	Nd	
Bbr	9.8[a]	25	9.8[a]	23	9.4[a]	12	9.3[a]	13	Nd	
PAD	ND			ND		ND		ND	Nd	
pBiBIF	ND			ND		ND		ND	Nd	
	Fermentation F2[ab]									
	M-10		EPS-11.5		FOS-10		M-11.5		Faeces	
	FISH counts	%	FISH counts	%	FISH counts	%	FISH counts	%	FISH counts	%
Eub338	10.5		10.6	10.6	10.5	10.9				
Bif164	10.4[a]	72[b]	10.4[a]	65[b]	10.5[a]	76[b]	10.4[a]	80[c]	10.8	90[c]
Blong209	10.0[a]	43	10.1[a]	49	10.4[b]	76	10.2[ab]	67	10.1	20
Bbr	9.8[a]	29	9.9[a]	31	9.7[a]	17	9.6[a]	17	10.5	50
PAD	9.9[a]	33	9.8[a]	28	9.8[a]	19	9.7[a]	22	ND	
pBiBIF	9.1[a]	5	9.0[a]	4	9.0[a]	3	9.0[a]	4	ND	

[a]Mean values of the two last days of each pseudo-steady state expressed in Log10 (cell number per mL).
[b]Effect of carbohydrate substrates on bifidobacteria populations: values with different letters in a row are significantly different with the Tukey–Kramer HSD test ($P < 0.05$).
[c]Percentage representing the proportion of bifidobacteria compared with the total bacterial population measured by FISH with the probe Eub338.
ND, not detected, value below the detection limit of the method [6 Log10 (cell number per mL)].
Nd, not determined.

(Table 6). During F1, the total SCFA concentration in all three reactors increased in the order: EPS-10, M-10, M-13, FOS-13. The decrease in total SCFA in EPS-10 was essentially the result of a decrease in acetic acid production. The high SCFA production for FOS-13 in R1 was associated with high acetic and propionic acid concentrations. FOS-13, M-10 and EPS-10 induced a significant decrease in isoacids in R3 (DCS) compared with M-13.

During F2, the total SCFA concentration increased in all three reactors in the order: M-10, EPS-11.5, FOS-10, M-11.5. In R1, FOS-10 and M-11.5 showed significantly higher concentrations of propionic and butyric acids compared with M-10. The highest concentration of butyric acid was measured in R1 during FOS-10. In R2, compared with M-10, the increase in total SCFA was mainly the result of an increase of acetic acid production for EPS-11.5, and of both acetic and propionic acids for FOS-10 and M-11.5. In R3, the total SCFA production for FOS-10 and M-11.5 was significantly higher than for M-10 and EPS-11.5, mainly as a result of increased acetic acid production. During F2, no significant changes of isoacid concentration were observed for all conditions and reactors.

In both fermentations, ammonia concentration increased from R1 to R3 for all tested periods (Table 7). Compared with M-13, FOS-13 significantly decreased ammonia production in R2 and R3 during F1, whereas no differences were observed between EPS-11.5 and M-11.5.

In F2, significant differences in ammonia production were only detected in R1 (PCS) (Table 7). The lowest concentrations of ammonia were measured for FOS-10 and M-11.5. EPS-11.5 also showed a significantly lower concentration of ammonia compared with M-10.

Analysis of EPS in EPS powder and fermentation samples

The content in low-molecular-weight sugars of the EPS powder was estimated from the carbohydrate concentration measured by phenol-sulphuric assay in the filtrate at the end of the purification procedure, and was $1.4 \pm 0.2\%$ and $1.1 \pm 0.1\%$ for the two powder preparations used for F1 and F2. The protein content for the two powders was $5.2 \pm 1.1\%$, as measured by the Lowry test. The EPS concentration in the fresh media used for EPS-10 and EPS-11.5 was 1481 ± 107 and $1496 \pm 119\,mg\,L^{-1}$, respectively. Residual EPS concentrations in the effluent from R3 (DCS) were 1476 ± 10 and $1469 \pm 15\,mg\,L^{-1}$, respectively, for EPS-

Table 6. Effect of medium carbohydrate composition on short-chain fatty acid and isoacid concentrations in effluents from the three reactors for two colonic fermentations, F1 and F2

	Fermentation 1[ab] (mM)			
	M-13	FOS-13	M-10	EPS-10
PCS (R1)				
Total SCFA	140.9[a]	166.5[a]	108.8[b]	97.1[b]
Acetate	96.0[ab]	108.3[b]	77.7[a]	53.4[c]
Propionate	25.6[a]	35.5[b]	16.4[c]	24.1[a]
Butyrate	19.3[a]	22.7[a]	14.7[a]	19.6[a]
Isoacids	10.1[a]	12.1[a]	10.3[a]	10.5[a]
TCS (R2)				
Total SCFA	161.1[a]	197.4[b]	136.3[a]	104.1[c]
Acetate	102.5[a]	118.4[a]	84.3[b]	50.9[c]
Propionate	33.1[a]	45.7[b]	26.0[c]	28.4[ac]
Butyrate	25.5[a]	33.3[a]	26.0[a]	24.8[a]
Isoacids	17.9[a]	20.7[a]	17.5[a]	10.4[a]
DCS (R3)				
Total SCFA	184.8[a]	192.8[a]	146.1[b]	130.1[c]
Acetate	114.9[a]	119.7[a]	94.0[b]	65.6[c]
Propionate	41.3[a]	42.1[a]	25.2[b]	34.2[ab]
Butyrate	28.6[a]	31.0[a]	26.9[a]	30.3[a]
Isoacids	22.0[a]	13.1[b]	15.9[b]	16.4[b]
	Fermentation 2[ab] (mM)			
	M-10	EPS-11.5	FOS-10	M-11.5
PCS (R1)				
Total SCFA	118.6[a]	125.2[ab]	137.4[b]	138.9[b]
Acetate	92.7[a]	97.9[a]	102.4[a]	108.6[a]
Propionate	20.4[a]	19.8[a]	26.9[b]	22.6[ab]
Butyrate	5.5[a]	7.5[b]	8.2[c]	7.7[bc]
Isoacids	3.6[a]	3.6[a]	3.6[a]	4.0[a]
TCS (R2)				
Total SCFA	141.4[a]	150.4[b]	159.1[b]	171.5[c]
Acetate	104.4[a]	111.4[ab]	113.3[b]	126.3[c]
Propionate	25.5[a]	26.7[a]	32.1[b]	31.8[b]
Butyrate	11.5[a]	12.4[a]	13.6[a]	13.4[a]
Isoacids	10.2[a]	10.6[a]	10.2[a]	13.4[a]
DCS (R3)				
Total SCFA	157.3[a]	158.3[ab]	167.9[b]	178.2[c]
Acetate	115.3[a]	115.3[a]	119.9[b]	131.4[c]
Propionate	29.0[a]	29.5[a]	33.1[a]	32.5[a]
Butyrate	13.0[a]	13.8[a]	13.9[a]	14.3[a]
Isoacids	12.6[a]	9.9[a]	10.6[a]	10.1[a]

PCS, proximal colon simulation; TCS, transverse colon simulation; DCS, distal colon simulation.

[a]Mean values of the three last days of each pseudo-steady state.

[b]Effect of carbohydrate substrates on organic acid production: values with different letters in a row are significantly different with the Tukey–Kramer HSD test ($P < 0.05$).

10 in F1 and EPS-11.5 in F2, which shows that EPS was not metabolized during intestinal fermentation.

Discussion

A number of chemostat systems have been designed to model the ecosystem of the human colon and to study bacterial composition and metabolism under controlled conditions. To solve the limitations of free-cell colonic models, we developed a new *in vitro* infant colonic model composed of a single-stage culture with immobilized infant faeces (Cinquin *et al.*, 2004), which was further developed into a three-stage chemostat model composed of three reactors in series simultaneously simulating the proximal,

Table 7. Effect of medium carbohydrate composition on ammonia production in the three reactors for two colonic fermentation experiments, F1 and F2

	Fermentation F1[ab] (mM)					Fermentation F2[ab] (mM)				
	M-13	FOS-13	M-10	EPS-10	*P*	M-10	EPS-11.5	FOS-10	M-11.5	*P*
PCS (R1)	7.7[ab]	4.8[a]	10.23[bc]	12.9[c]	< 0.005	9.1[a]	6.9[b]	1.7[c]	1.6[c]	< 0.0005
TCS (R2)	24.6[a]	14.4[a]	24.5[a]	23.3[a]	< 0.0005	29.4[a]	28.5[a]	29.7[a]	29.2[a]	
DCS (R3)	26.3[a]	16.5[b]	29.1[c]	35.4[d]	< 0.0005	33.5[a]	31.7[a]	32.3[a]	32.2[a]	

PCS, proximal colon simulation; TCS, transverse colon simulation; DCS, distal colon simulation.

[a]Mean values of the three last days of each pseudo-steady state.

[b]Effect of carbohydrate substrates on ammonia production: values with different letters in a row are significantly different with the Tukey–Kramer HSD test.

transverse and distal parts of the infant colon (Cinquin *et al.*, 2006). This immobilized-cell model was validated by microbiological analyses using both plate counts on selective media and molecular methods, as well as metabolic data (Cinquin *et al.*, 2004, 2006). Indeed, we showed that cell immobilization induced a high cell density in beads and fermentation medium, comparable with *in vivo* values, improved stability, and preserved bacterial diversity of the faecal inoculum over long culture periods. The maintenance of a stable community in all reactors can be explained by immobilized faecal microbiota growth, cell release from beads, and eventually growth of free cells in the bulk medium. In the present study, we used this new three-stage chemostat model with immobilized cells to investigate the prebiotic effects of EPS produced by *L. rhamnosus* RW-9595 M on infant faecal microbiota, in parallel with a well-characterized prebiotic, fructo-oligosaccharides (FOS), with two continuous culture experiments. In a previous paper (Cinquin *et al.*, 2006), we reported a detailed analysis of stabilization periods for F1 (M-13) and F2 (M-10).

In this study, replacement of maltodextrin in M-13 by FOS during F1 (FOS-13) gave increased numbers of lactobacilli, and of bifidobacteria to a lesser extent, while less desirable bacteria such as coliforms, clostridia and bacteroides showed decreased numbers in beads and fermentation effluents (Table 4). Similar effects have been reported for FOS for various *in vitro* (Michel *et al.*, 1998; Rycroft *et al.*, 2001a) and *in vivo* (Gibson *et al.*, 1995) studies in adults or following the ingestion of a prebiotic mixture of FOS and galacto-oligosaccharides in infants (Fanaro *et al.*, 2005). In our study, a larger increase in numbers was observed for lactobacilli (plus 0.8 to 1.2 Log10 for FOS-13 compared with M-13, $P < 0.05$), whereas only a slight increase in bifidobacteria was observed (plus 0.3 to 0.6 Log10, $P < 0.05$ only in R3) in the fermentation effluents from the three reactors for F1. This low effect of FOS on the number of bifidobacteria is probably a result of the high initial bifidobacteria counts measured in the M-13 control (9.6 Log10 CFU mL^{-1}). Kleessen *et al.* (2001) did not observe any bifidogenic effects of inulin on microbiota *in vivo* in rats associated with human faecal microbiota, probably because of the high bifidobacteria counts in the control. Indeed, Rycroft *et al.* (2001b) showed a clear correlation between the increase in bifidobacterial populations and their initial number, with a stronger effect for low initial levels of bifidobacteria. In contrast to F1, no stimulation of lactobacilli nor of bifidobacteria was observed during the substitution of maltodextrin in F2 (M-10) for FOS (FOS-10). This lack of stimulation, compared with F1, might be explained by the lower FOS concentration tested in F2 (7.5 vs. 9.8 g L^{-1}) combined with high initial counts of lactobacilli and bifidobacteria before FOS treatment (6.3 and 9.7 Log10 CFU mL^{-1} respectively). Moreover, the FOS effect is known to be dose-dependent, as observed in infants (Moro *et al.*, 2002) and adults (Bouhnik *et al.*, 1999).

Fluorescence *in situ* hybridization analysis confirmed the high numbers of bifidobacteria in both fermentations (10.1–10.5 Log10 cell number mL^{-1}). Close to 100% of the bifidobacterial community was recovered with the four species-specific probes tested (Table 5), while *B. longum* was the major population, representing 57–74% of the bifidobacterial community. Only *B. longum* and *B. breve* were detected in F1, whereas the four species were detected in F2, including *B. adolescentis* and *B. bifidum*, which were below the detection limit in the faecal inoculum. FOS did not lead to significant increases in total counts of bifidobacteria in either fermentation. In F2, however, FOS (FOS-10) changed the balance for bifidobacterial species, and specifically increased the numbers and proportion of *B. longum* compared with M-10 and EPS-11.5. This result is in agreement with data from Bielecka *et al.* (2002), who showed that *B. longum*, *B. infantis* and *B. animalis* were able to ferment FOS better than other bifidobacterial species. This effect was not observed in F1, probably because of the initially high proportion of *B. longum* present in the fermentation effluents before FOS treatment. In the present study, the high levels of bifidobacteria in both faecal samples and fermentation effluents were probably responsible for the lack of bifidogenic effect observed with FOS. However, despite the limited effect of FOS on total numbers of

bifidobacteria, the observed stimulation of lactobacilli and *B. longum* together with the decrease in coliforms are in agreement with published data (Gibson *et al.*, 1995; Bielecka *et al.*, 2002).

Moreover, FOS was well fermented by the bacterial community established in the continuous culture, and gave increases in SCFA production and decreases in ammonia. This supports other data in the literature (Gibson & Roberfroid, 1995) and confirms the good reactivity and validity of our system for modelling intestinal fermentations. However, the reduction in ammonia production was observed in all three reactors in F1, but was only observed in R1 for F2. This difference is probably related to the lower amounts of FOS tested in F2 (FOS-10 with 7.5 g L^{-1} of FOS) compared with F1 (FOS-13 with 9.8 g L^{-1} of FOS). Because FOS are easily metabolized by the microbiota, they might have been completely fermented in the first reactor during F2. In contrast with *in vitro* data obtained with adult faecal microbiota, which showed the butyrogenic effects of FOS on SCFA production (Rycroft *et al.*, 2001a), the increase in SCFA production in our study was obtained without modifying the butyrate ratio. This absence of butyrogenic effect during FOS fermentations has been observed *in vitro* with infant microbiota (Flickinger *et al.*, 2002), and may be associated with the reduced bacterial complexity of infant microbiota and with lower concentrations or the absence of butyrate producers compared with adult microbiota (Harmsen *et al.*, 2000; Cinquin *et al.*, 2006). It has been shown that the growth or activity of these bacteria can be stimulated by prebiotics and complex carbohydrates (Pryde *et al.*, 2002), and that infants have a lower capacity to produce butyrate than adults (Parrett *et al.*, 1997).

The central aim of this study was to investigate the potential prebiotic effects of EPS produced by *L. rhamnosus* RW-9595 M on infant bacterial microbiota compared to FOS, a well-known prebiotic. In F1, the substitution of 1.5 g L^{-1} of maltodextrin (M-10) by the same amount of EPS (EPS-10) induced a decrease in total SCFA concentrations, believed to be associated with a decrease in acetate ratios, and increases in propionate and butyrate ratios. Similar effects were reported for long compared with short retention times during *in vitro* colonic fermentations by Macfarlane *et al.* (1998). This suggests that a lower fermentable carbohydrate concentration was available for EPS-10 compared with M-10. This was confirmed by an increase in ammonia concentrations in EPS-10 compared with M-10. Indeed, ammonia, which is produced during protein breakdown, reflected the increase in the protein/carbohydrate ratio between M-10 and EPS-10. These results were confirmed during F2 by the lack of stimulation of SCFA production observed in EPS-11.5 (M-10 plus 1.5 g L^{-1} EPS) compared with M-10, whereas M-11.5 (M-10 plus 1.5 g L^{-1} maltodextrin) resulted in SCFA increases. Data from both fermentation experiments suggest that EPS was not metabolized by the infant microbiota. Moreover, quantification of residual EPS in the fermented media confirmed that the EPS used in this study was not fermented.

In F1, increases in the facultative anaerobic populations observed for EPS-10 compared with M-10 could be a result of reductions of fermentable carbohydrates in the medium or of specific effects of EPS. A second fermentation, F2, was carried out to differentiate between these effects. The addition of EPS in medium M-10 (EPS-11.5) led to decreases in lactobacilli and staphylococci counts compared with M-10 in all three reactors, although the amount of fermentable carbohydrates remained the same.

Data obtained with FOS in this study are in agreement with previous published studies, supporting the validity of the *in vitro* model using immobilized faecal bacteria. Moreover, we showed that the balance of different bifidobacterial species could be modified by FOS, with a specific stimulation of *B. longum*, although no effect was detected on the total bifidobacterial population. To our knowledge, this is the first time that the effects of different polysaccharide substrates on bifidobacterial species have been studied by FISH in a complex bacterial ecosystem. EPS produced by *L. rhamnosus* RW-9595 M was not metabolized by infant microbiota and did not exert any prebiotic effects. On the contrary, our data suggest that this EPS has a detrimental effect on bacterial balance by decreasing lactobacilli numbers.

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