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Microbial Ecology

Immobilization of Infant Fecal Microbiota and Utilization in an *in vitro* Colonic Fermentation Model

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Abstract

Bacteria isolated from infant feces were immobilized in polysaccharide gel beads (2.5% gellan gum, 0.25% xanthan gum) using a two-phase dispersion process. A 52-day continuous culture was carried out in a single-stage chemostat containing precolonized beads and fed with a medium formulated to approximate the composition of infant chyme. Different dilution rates and pH conditions were tested to simulate the proximal (PCS), transverse (TCS), and distal (DCS) colons. Immobilization preserved all nine bacterial groups tested with survival rates between 3 and 56%. After 1 week fermentation, beads were highly colonized with all populations tested (excepted *Staphylococcus* spp. present in low numbers), which remained stable throughout the 7.5 weeks of fermentation, with variations below 1 log unit. However, free-cell populations in the circulating liquid medium, produced by immobilized cell growth, cell-release activity from gel beads, and free-cell growth, were altered considerably by culture conditions. Compared to the stabilization period, PCS was characterized by a considerable and rapid increase in *Bifidobacterium* spp. concentrations (7.4 to 9.6 log CFU/mL), whereas *Bifidobacterium* spp., *Lactobacillus* spp., and *Clostridium* spp. concentrations decreased and *Staphylococcus* spp. and coliforms increased during TCS and DCS. Under pseudo-steady-state conditions, the community structure developed in the chemostat reflected the relative proportions of viable bacterial numbers and metabolites generally encountered in infant feces. This work showed that a complex microbiota such as infant fecal bacteria can be immobilized and used in a continuous *in vitro* intestinal fermentation model to reproduce the high bacterial concentration and bacterial diversity of the feces inoculum, at least at the genera level, with a high stability during long-term experiment.

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Introduction

The gut microbiota is part of a complex ecosystem having significant impact on human health, particularly for infants in whom perturbation of gut microbiota can lead to major intestinal disorders and infections [45]. Ethical and accessibility problems limit *in vivo* studies of gut microbiota, especially in healthy infants. Various *in vitro* approaches such as batch culture and single- or multi-stage chemostats have been used to study adult and infant colonic microbiota to a lesser extent [9, 19]. Compared with batch cultures, continuous culture models are particularly well suited for ecological studies [44]. However, all models are based on free-cell suspension cultures. These may present stability problems such as loss of less competitive bacteria during experimental trials [16, 53], long stabilization periods over 3 weeks [8, 16], washout due to short retention times [8], and difficulty in creating cell-wall growth in a single-stage chemostat [16]. As a result, most experiments do not exceed 4 weeks, which can prevent the testing of different parameters successively [9, 36, 48]. Moreover, these models poorly reproduce the colonic ecosystem, characterized by bacteria in the immobilized state, growing in intimate associations on the surface of food particles [35], or forming biofilms on the intestinal epithelium [43]. Indeed, stirring of free-cell cultures must be slow enough to allow wall growth in order to obtain a culture composition similar to *in vivo* gut microbiota [53]. Even so, when steady state is reached, the total bacterial concentration ($<10^{10}$ CFU/mL) in free-cell cultures is lower than that (10^{10}–10^{11} CFU/g) observed in intestinal contents [34]. In order to more closely simulate conditions in the large intestine and increase bacterial density, Minekus et al. [40] de-

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veloped a proximal colon system with free cells and metabolites and water absorption.

To solve problems associated with free-cell systems and more closely mimic the intestinal conditions in which bacteria are in close association with particulate material [35], we propose the use of cell immobilization and continuous fermentation. Recently, enhanced cell-to-cell signaling and cell-matrix interactions leading to coordinated behavior of immobilized microorganisms have been reported [49]. Cell immobilization has been applied to numerous food fermentations using a wide variety of microorganisms, including bacteria, yeasts, molds, and plant cells [21, 28]. The most widely used immobilization technique is the entrapment of cells within a porous polysaccharide matrix. When immobilized cells are transferred in a growth medium, limitations on the diffusion of substrates and products result in the formation of a high-cell density layer (typically ranging from 10^{10} to 5×10^{11} CFU/mL gel beads) extending from the bead surface to a radial depth where lack of substrate or accumulation of inhibitory product (e.g., organic acids) and local physicochemical conditions prevent cell growth [5, 12, 28]. Cell release from gel beads in the liquid medium occurs spontaneously because of active cell growth in the high-biomass-density peripheral layer. Major advantages were demonstrated with lactic acid bacteria and bifidobacteria for this technology compared with conventional free-cell fermentations, including stable strain ratios with mixed cultures and prevention of washout during long-term continuous culture, reduction of susceptibility to contamination and bacteriophage attack, enhancement of plasmid stability, protection of cells from shear forces in the stirred reactor, and continuous and uniform inoculation of the bulk medium [5, 28].

In this study, we hypothesized that cell immobilization could improve *in vitro* colonic fermentation models by better mimicking the colonic environment and lead to higher cell density and stability over long periods. As a first step, this feasibility study aims to demonstrate the use of immobilized fecal microbiota to simulate colonic fermentation. The aim of the study was to (1) immobilize complex fecal microbiota generally reproducing the environmental composition of infant intestinal microbiota *in vitro,* and (2) study changes in composition and activity of the colonic microbiota in the *in vitro* model using immobilized cells operated over a long time period of 54 days with different conditions simulating those of the different colonic segments.

Materials and Methods

Fecal Sample Collection and Preparation. Fecal samples were collected from four healthy 4- to 6-month-old infants to test the effects of immobilization on fecal microbiota, and from one healthy 180-day-old infant for continuous culture. All infants had never received antibiotics and were in early weaning onto cereal. Fresh feces (~2 g) were scraped from diapers, transferred to a tube containing 7 mL of sterile, prereduced peptone water (0.1%, pH 7), placed in an anaerobic jar (Anaerojar, Oxoid, Hampshire, England) kept at 4°C, and immediately delivered to the laboratory. Fecal suspensions were weighed and the volume was adjusted with peptone water to obtain a final fecal concentration of 20% (w/v). They were then homogenized and gently centrifuged (700 g for 1 min) to remove large particles. The procedure was completed within 2 h after defecation.

Culture Medium. A culture medium based on the composition described by Macfarlane et al. [34] was modified in order to simulate chyme produced from an infant diet. Bile salt concentration was reduced to reflect the lower level of secretion in infants [54]. Yeast extract, casein, peptone, and tryptone concentrations were reduced, and hydrolyzed whey proteins were added to the medium. We chose to add hydrolyzed whey proteins for technical reasons since they were hardly soluble in the culture medium. Peptone and tryptone as yeast extract are classical compounds and were added to culture medium to ensure bacterial growth. Starch, maltodextrin, and lactose replaced complex fibers, while mucin concentration was not modified.

The composition of the culture medium was calculated from the composition of a standard infant formula supplemented with rice starch (Enfamil A.R., Mead-Johnson Nutritionals, Evansville, IN, USA). This formula is composed of 74 g/L carbohydrates (lactose, 57%, pregelatinized rice starch, 30% and maltodextrin, 13%), and 16.9 g/L proteins (whey:casein ratio of 18:82). For experimental convenience, the whey:casein ratio was adjusted to 40:60, which is widely used in infant formula. Digestibility indices from the literature were applied to each substrate to obtain a carbohydrate:nitrogen compounds ratio of 58:42 for the infant ileal chyme-simulating culture medium (Table 1). The total carbohydrate concentration (13 g/L) of the culture medium was chosen to provide enough substrate to promote bacterial growth [34]. The total nitrogen compound concentration was thus set at 9.4 g/L, which included nitrogen compounds originating from casein and whey protein digestion (Table 1), tryptone, and peptone added to the medium at 0.5 g/L each. Although yeast extract and mucin (glycoprotein) contributed to nitrogenous compounds and carbohydrates, they were ignored in the ratio calculation since they simulated the contribution of endogenous secretions.

The culture medium thus consisted of the following constituents (grams per liter of distilled water), NaCl: 4.5; KCl: 4.5; $MgSO_4$, $7H_2O$: 1.25; $CaCl_2$, $2H_2O$: 0.15; K_2HPO_4: 0.5; $NaHCO_3$: 1.5; $FeSO_4$, $7H_2O$: 0.005; cysteine: 0.8; bile salts: 0.05; mucin (porcine gastric type II,

Table 1. Estimation of exogenous substrates available for colonic fermentation in infants

Infant milk formula[a]				*Ileal chyme simulation*		
Class compound	*Ingredients*	*Proportion (%)*	*Digestibility indices (%)*	*Undigested ingredient*[b] *(g/L)*	*Undigested ingredient proportion*[c] *(%)*	*Ingredient and concentrations in the culture medium*[d] *(g/L)*
Carbohydrates (74 g/L)	Rice starch	30	95 (6)	1.11	52	6.8
	Maltodextrin	13	98 (6)	0.19	9	1.2
	Lactose	57	98 (26)	0.84	39	5
N-compounds (16.9 g/L)	Casein	60	98 (15)	0.20	13	1.2
	WPH[e]	40	80 (31)	1.35	87	7.2

[a]Enfamil A.R., MeadJohnson Nutritionals, Evansville, IN, USA, the whey:casein ratio was adjusted to 40:60.
[b]Estimation of undigested material concentrations available for colonic fermentation.
[c]Proportions calculated as follows: undigested ingredient (g/L)/undigested total carbohydrates (2.1 g/L) or N-compounds (1.5 g/L).
[d]The total carbohydrate concentration (13 g/L) of the culture medium was based on data from Macfarlane et al. [34]. The amount of nitrogen compound was adjusted to give a 58:42 ratio of undigested carbohydrate:nitrogen compound that could reach the colon.
[e]Whey protein hydrolysate.

Sigma, St Louis, MO, USA): 4.0; Tween 80: 1.0; hemin: 0.01; yeast extract: 2.5; peptone: 0.5; tryptone: 0.5; casein (Difco, Detroit, MI, USA): 1.2; whey protein hydrolysate (BioZate 1, Davisco, Le Sieur, MN, USA): 7.2; soluble rice starch (Sigma): 6.8; maltodextrin: 1.2; lactose: 5.0. In addition, 0.5 mL per liter of culture medium of a vitamin solution described by Gibson and Wang [20] was added to the culture medium. Lactose, cysteine, and vitamin solutions, were sterilized by filtration (0.2 μm, Sartorius, Gottingen, Germany) and added separately to the autoclaved medium (15 min, 121°C).

Immobilization Technique. The mixed gel used for immobilization was composed of gellan gum (2.5%, Gelrite, Sigma) and xanthan gum (0.25%, w/v) (Sigma). The dried powders were suspended in 200 mL of preheated distilled water (90°C) containing sodium citrate (0.2%) and mixed in a blender until dissolved (1 min). The polymer solution was then autoclaved for 15 min at 121°C just prior to use for immobilization.

The immobilization procedure was based on a dispersion process in a two-phase system, as described by Lamboley et al. [30] for kappa-carrageenan/locust bean gum gel bead preparation. The autoclaved polymer solution was cooled to 43°C and inoculated aseptically with the fecal inoculum (2%). After inoculation, the polymer solution was stirred into freshly autoclaved (15 min at 121°C) hydrophobic phase (commercial canola oil) at 43°C to obtain a suspension of aqueous droplets in oil. Gel beads were formed by cooling the suspension. After separation and washing, beads were hardened by soaking for 30 min in $CaCl_2$ (0.1 M). Beads with diameters in the 1.0–2.0 mm range were selected by wet sieving and used for fermentation. The entire process was completed in aseptic conditions within 1 h.

Fermentation Procedures. Batch fermentations were first carried out for bead colonization in a custom-stirred flat-bottom glass bioreactor (working volume: 100 mL) containing 30% (v/v) of freshly inoculated beads. During the colonization step (days 1 and 2), the fermented medium was aseptically replaced by nonfermented medium every 12 h. The fermenter was maintained at 37°C by circulating thermostated water in the double wall. Stirring was provided by a magnetic bar; and anaerobic conditions were maintained by a continuous flow of pure CO_2. The pH was controlled at 6.2 with a pH-meter titrator (PHM84, TTT80 Titrator, Radiometer, Copenhagen, Denmark) connected to a magnetic valve dispensing NaOH (4 N) by gravity.

Continuous fermentation was carried out in the same fermenter connected to a stirred feedstock vessel containing sterile culture medium at 4°C and to an effluent receiving vessel. Continuous feeding and harvesting of medium was carried out using a peristaltic pump (Minipuls 3, model M312, Gilson, Villiers le Bel, France), whereas the beads were retained in the fermenter. The pH was controlled by addition of 4 N NaOH during the stabilization period (SP) and 6 N NaOH during proximal colon (PCS), transverse colon (TCS), and distal colon (DCS) conditions, respectively. During the PCS period, problems arose with pH control because of a defective electrode, resulting in pH variation from 5.7 to 7.5 between day 19 and day 27.

The culture was performed for 54 days (2 days of batch culture, plus 52 days of continuous culture). During the 16-day stabilization period, the mean retention time was set at 12.5 h by adjusting the feed flow rate to 8 mL/h, and pH was controlled at 6.2 to complete bead colonization. Mean residence time and pH were then set at 4 h and 5.7 for 10 days (PCS); 8 h and 6.2 for 10 days (TCS);and finally 12.5 h and 6.8 for 6 days (DCS). Effluent samples (4 mL) were collected daily for bacterial enumeration and metabolite analyses, and 0.5 g to 1 g of gel beads was collected weekly for bacterial enumeration throughout the 54-day run. The pseudo-steady state for

each condition (SP, PCS, TCS and DCS) was considered reached when bacterial populations in the reactor effluent did not change by more than 0.5 log units during 4 consecutive days.

The experiment was reproduced with fecal material obtained from one other infant (6-month-old) to confirm the results obtained. Again, this infant had never received antibiotics and was in early weaning onto cereal. Because the observed effects were similar, only the results from the first experiment are reported here.

Bacteriological Analyses. All bacteriological analyses were performed within 1 h after 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 [27]. Total and facultative anaerobes were enumerated using Wilkins-Chalgren agar (Oxoid, Basingstoke, Hampshire, England) and nutrient agar (Oxoid), respectively. Different selective media were used to enumerate the nine bacterial populations: Beerens agar for bifidobacteria [2], bacteroides mineral salt-based medium for *Bacteroides fragilis* group [33], sulfite-polymyxin-milk agar for clostridia [10], azide blood agar base for Gram-positive cocci (Oxoid), Mac Conkey agar no. 2 for coliforms (Oxoid), LAMVAB agar for lactobacilli [23], and mannitol salt agar for staphylococci (Oxoid). Plates were incubated aerobically or in anaerobic jars at 37°C for up to 4 days. Cell counts were expressed as log colony-forming units (CFU) per gram (wet weight) of feces, per mL of fermentation medium, or per g (wet weight) gel beads.

Viable cell count determinations in beads were performed by dissolving approximately 0.5 g of accurately weighed beads in 1% EDTA solution at pH 7 using a stomacher (Lab Blender 400, Bury St Edmunds, England) for 5 min. Preliminary tests were done to show that this step did not reduce the bacterial numbers. Bacterial survival rates in freshly prepared beads were expressed with respect to initial counts in fecal samples (log CFU/g feces).

HPLC Analyses. Samples of fermentation medium (1 ml) were diluted in the mobile phase (H_2SO_4, 0.0065 N), centrifuged (10,000 g for 10 min at 4°C), and filtered with Acrodisc LC 13 mm syringe filters with 0.2-μm PVDF (hydrophilic polyvinyldene fluoride) membrane (Pall, Ann Arbor, MI, USA) in the vials, which were immediately sealed. The filtrate samples were stored at −20°C until analysis. Short-chain fatty acids (SCFA), branched-chain fatty acids (BCFA: isobutyrate and isovalerate), valerate, and lactate concentrations were determined by HPLC (Waters, Milford, MA, USA) equipped with Agilent ChemStation Version 6.2, using the method described by Doyon et al. [14] and a differential refractometer (Model R410, Waters). The analysis was performed at a flow rate of 0.4 mL/min at 25°C, with an injection volume of 100 μL. Each analysis was done in duplicate. The mean metabolite concentrations were expressed in mmol/L (mM).

Statistical Analyses. A one-way analysis of variance (ANOVA) was performed using Statview F-4.11 (Abacus Concepts Inc., Berkeley, CA, USA) to test the effects of pH and dilution rates on bacterial populations and metabolite concentrations measured during pseudo-steady-state periods (mean of 4 successive days) in effluent samples for each tested condition (SP, PCS, TCS, and DCS). When significant differences were found below the probability level of 0.05, treatment means were compared using the Fisher test. Statistical significance was accepted at $P < 0.05$ level. Correlations among parameters were also analyzed with the Statview F-4.11 package. Beads were only sampled once a week for viable cell count determinations to avoid large changes in bead volume due to multiple sampling during the 54-day continuous fermentation. Therefore only means of duplicate plating in beads are reported for each sampling time.

Results

Effects of Immobilization on Fecal Bacterial Populations. In fecal samples used to prepare the inoculum for the continuous fermentation experiment, bifidobacteria were the predominant organisms (10.2 log CFU/g), followed by coliforms (8.8 log CFU/g), clostridia, and lactobacilli (8.1 and 7.8 log CFU/g, respectively). Staphylococci showed the lowest concentration (4.8 log CFU/g) (Table 2). The fecal microbiota from the other four infants were comparable with the one used to inoculate the continuous fermentation.

Immobilization affected all bacterial groups, which decreased by similar amounts (0.5 to 1 log unit). *Clostridium* spp. showed a slightly larger decrease (1.5 log unit), while the *Bacteroides fragilis* group was not detected in fresh beads after immobilization. The detection threshold of the method for beads (4 log CFU/g beads corresponding to approximately 6 log CFU/g feces) did not allow the quantification of immobilization effects on staphylococci because of their low initial concentration (4.8 log CFU/g) in feces (Table 2). The bacterial groups studied showed survival rates between 11 and 51%, with the exception of *Clostridium* spp. with only 3% of cells surviving gel bead entrapment. Similar survival rates (12–56%) were obtained after immobilization of fecal bacteria from the other four infants.

Changes in Immobilized and Free-Cell Populations During Continuous Culture. After 1 week of culture (SP, day 8), gel beads were highly colonized (Table 2).

Table 2. Viable bacterial numbers in the feces used for inoculum preparation and in gel beads during continuous culture

	Viable bacterial numbers[b]							
Period[a]	*Feces*	*Immo*	*SP*	*SP*	*pH var*	*PCS*	*TCS*	*DCS*
Sampling day	*0*	*0*	*8*	*15*	*23*	*35*	*45*	*54*
Total anaerobes	10.2	7.3	9.6	9.5	10.0	10.5	9.5	9.6
Bifidobacterium spp.	10.2	7.5	8.7	8.3	9.1	9.0	8.3	7.3
Bacteroides fragilis group	7.5	<4	5.3	8.5	8.6	9.0	8.5	8.3
Clostridium spp.	8.1	4.0	6.0	5.5	7.1	6.3	6.0	5.0
Facultative anaerobes	8.9	5.8	9.6	8.6	9.9	9.6	9.5	9.3
Coliforms	8.8	5.4	7.7	7.6	9.0	9.2	8.5	9.3
Gram-positive cocci	7.8	5.0	8.5	8.7	9.4	9.2	8.9	9.4
Lactobacillus spp.	7.8	4.9	7.2	7.6	7.3	7.5	7.2	6.6
Staphylococcus spp.	4.8	<4	<4	<4	<4	<4	5.6	5.0

Reported data are means from duplicate plating of single sample of feces or beads during pseudo-steady states.
[a]Immo: fresh beads after immobilization; SP: stabilization period; pH var: uncontrolled pH; PCS: proximal colon simulation; TCS: transverse colon simulation; DCS: distal colon simulation.
[b]Log (CFU/g of feces (wet weight) or beads).

Stable values were reached within 1 week for all bacterial populations tested except for the *Bacteroides fragilis* group, which took ~2 weeks (SP, day 15) to stabilize at 8.5 log CFU/g beads and staphylococci, which remained below the detection threshold of the method for beads (4 log CFU/g beads) (Table 2).

Most of the bacterial population levels and their relative proportions in beads were only slightly affected by changes of pH and residence time during PCS, TCS, and DCS, with variations below 1 log unit (Table 2). Facultative anaerobes, clostridia, and coliforms were the most affected, with maximal variations of 1.3, 1.6, and 1.4 log units, respectively, between two successive conditions. Staphylococci were detected (5.6 log CFU/g beads) only after day 45. Bifidobacteria, *Bacteroides fragilis* group, coliforms, and Gram-positive cocci were the predominant populations in beads throughout the culture experiment, with numbers higher than 8 log CFU/g beads (Table 2).

Total bacterial population showed no variation during the 54-day culture with an average concentration of 9.9 ± 0.02 log CFU/mL in liquid medium. However, the subpopulations tested were strongly affected by culture conditions (Figs. 1 and 2). After 48 h of batch fermentation (day 2), the culture medium contained all the populations in high numbers, except for staphylococci, which were not detected by the method (detection threshold of 3 log CFU/mL in the culture effluent). The populations then decreased when continuous culture was started on day 3 and increased progressively to reach a pseudo-steady state on day 18 of SP. Staphylococci were detected in the fermented medium after day 13 of SP, at numbers ranging from 3.9 to 4.4 log CFU/mL.

During the PCS period, problems arose with pH control because of a defective electrode, resulting in pH variation from 5.7 to 7.5 (days 19–27). It became apparent that the free-cell bacterial populations were very sensitive to pH changes at a constant dilution rate of 0.25

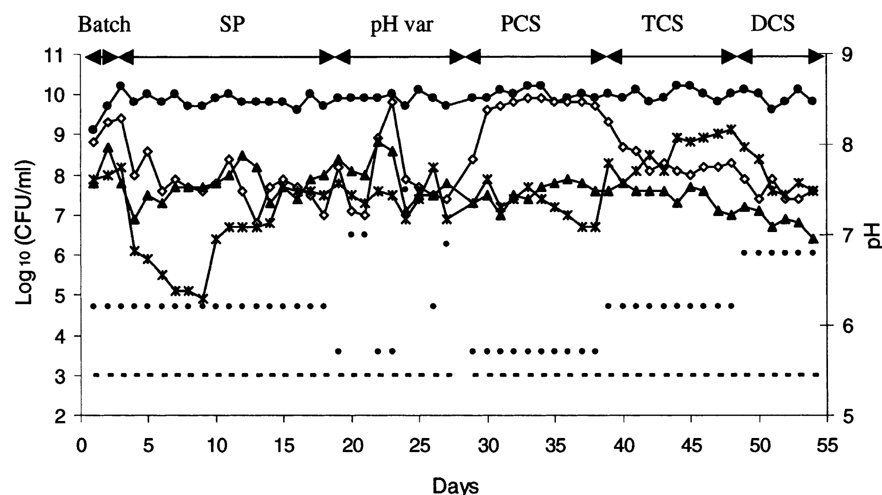

Figure 1. Change in anaerobe numbers in the fermented medium as a function of culture conditions. Total anaerobes (●), bacteroides (∗), bifidobacteria (◇), clostridia (▲), pH (•), detection limit of the enumeration method in the effluent (—). SP: stabilization period (pH 6.2, 0.08 h^{-1}); PCS: proximal colon simulation (pH 5.7, 0.25 h^{-1}); TCS: transverse colon simulation (pH 6.2, 0.125 h^{-1}); DCS: distal colon condition (pH 6.8, 0.08 h^{-1}); pH var: uncontrolled pH period.

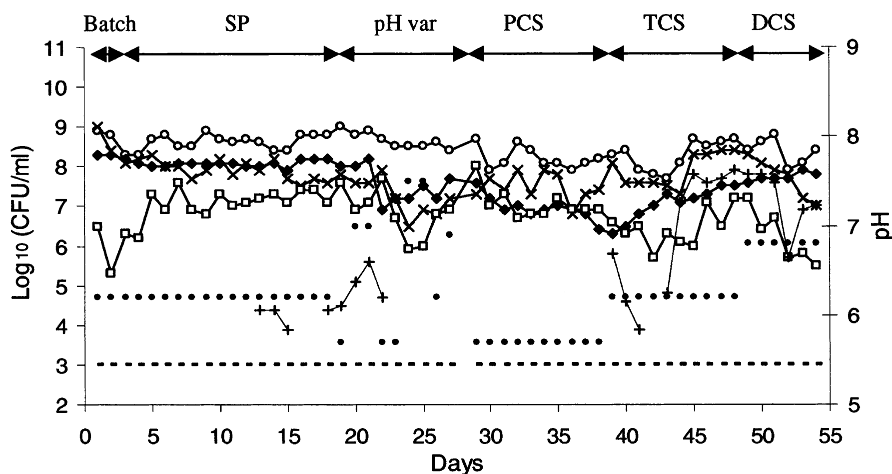

Figure 2. Change in facultative anaerobe numbers in the fermented medium as a function of culture conditions. Facultative anaerobes (○), coliforms (♦), Gram-positive cocci (×), lactobacilli (□), staphylococci (+), pH (•), detection limit of the enumeration method in the effluent (—). SP: stabilization period (pH 6.2, 0.08 h^{-1}); PCS: proximal colon simulation (pH 5.7, 0.25 h^{-1}); TCS: transverse colon simulation (pH 6.2, 0.125 h^{-1}); DCS: distal colon condition (pH 6.8, 0.08 h^{-1}); pH var: uncontrolled pH period.

h^{-1}. Acidic pH accelerated growth of *Bifidobacterium* spp. (7 vs 9.8 log CFU/mL) and brought a rapid decrease in coliform counts (8.2 to 6.9 log CFU/mL), whereas a more basic pH (6.9–7.5) decreased *Lactobacillus* spp. (7.7 to 5.9 log CFU/mL) and *Bifidobacterium* spp. (9.8 to 7.0 log CFU/mL) counts and increased bacteroides (6.9 to 8.2 log CFU/mL) and staphylococci (<3 to 5.6 log CFU/mL) numbers (Figs. 1 and 2). On day 28 the pH electrode was changed under aseptic conditions. Compared to the stabilization period, PCS (days 28–38) was characterized by a rapid and considerable increase in *Bifidobacterium* spp. numbers during the 72-h period following the change in conditions (Fig. 1), from 7.4 (day 27) to 9.6 log CFU/mL (day 30). When conditions were changed from PCS to TCS and DCS, *Bifidobacterium* spp., *Lactobacillus* spp. and *Clostridium* spp. numbers decreased while *Staphylococcus* spp. and coliforms increased (Figs. 1 and 2). The coliform concentration was equal to that for *Bifidobacterium* spp. during DCS (7.8 to 7.6 log CFU/mL, respectively). The *Bacteroides fragilis* group increased during TCS, in which they became the predominant population, but decreased during DCS. The total anaerobe free-cell population reached very high numbers (~10 log CFU/mL) and was not affected by the tested conditions (Fig. 1).

When comparing the bacterial populations between feces and culture effluent samples ($n = 4$) under pseudo-steady-state conditions (Table 3), PCS was the closest to the inoculum. There was no significant difference between total anaerobes concentrations in PCS, TCS, and DCS compared with the fecal inoculum (9.9 ± 0.05; 10.0 ± 0.09, and 9.8 ± 0.12, respectively, vs 10.2 log CFU/mL). However, the coliform concentration in the effluent was significantly lower ($P < 0.0001$) during PCS compared with the inoculum (6.8 ± 0.1 vs 8.8 log CFU/mL). During TCS, the *Bacteroides fragilis* group and staphylococci counts were significantly higher ($P < 0.0001$) than in the inoculum, where coliforms and bifidobacteria were significantly lower. During DCS, none of the bacterial groups measured in this study was dominant in the fermented medium (Figs. 1 and 2), whereas bifidobacteria were largely dominant in the inoculum.

Table 3. Bacterial concentrations in the feces used for inoculum preparation and in effluent at different pseudo-steady states during continuous culture

Sampling days	*Inoculum*[1]	*SP*[2] 16–18	*PCS*[2] 35–38	*TCS*[2] 45–48	*DCS*[2] 51–54	*SEM*	*P*
Total anaerobes	**10.2**	**9.8**	**9.9**	**10.0**	**9.8**	**0.04**	**0.2**
Bifidobacterium spp.	10.2a	7.5b	9.8a	8.2c	7.6b	0.27	<0.0001
Bacteroides fragilis group	7.5a	7.6a	6.9a	9.0b	7.6a	0.19	<0.0001
Clostridium spp.	8.1a	7.8a	7.7a	7.4ab	6.7b	0.12	<0.0001
Facultative anaerobes	**8.9a**	**8.7a**	**8.1b**	**8.6ac**	**8.3bc**	**0.08**	**0.01**
Coliforms	8.8a	8.1a	6.8b	7.4c	7.8ac	0.15	<0.0001
Gram-positive cocci	7.8	7.6	7.8	8.4	7.5	0.12	0.5
Lactobacillus spp.	7.8a	7.2a	7.0a	6.7ab	5.9b	0.16	0.001
Staphylococcus spp.	4.8a	3.9a	≤ 3	7.8b	6.8b	0.49	<0.0001

Values with different letters in a row significantly differ ($P < 0.05$).
[1]Bacterial concentration (log CFU/g) in feces ($n = 1$).
[2]Mean bacterial concentration (log CFU/mL) in effluent samples ($n = 4$) harvested daily during pseudo-steady states.

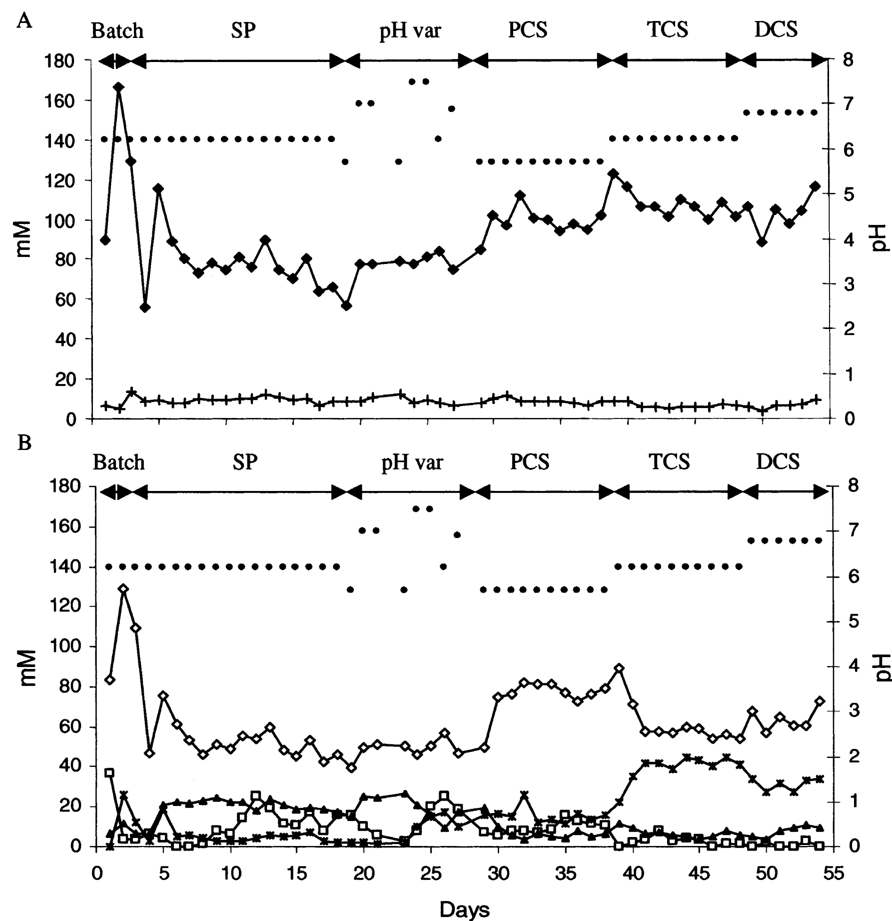

Figure 3. SCFA, BCFA, and lactate concentrations in the fermented medium; (A) total SCFA (●), BCFA (+), pH (•); (B) acetic acid (◇), butyric acid (▲), lactic acid (□), propionic acid (∗), pH (•). SP: stabilization period (pH 6.2, 0.08 h^{-1}); PCS: proximal colon simulation (pH 5.7, 0.25 h^{-1}); TCS: transverse colon simulation (pH 6.2, 0.125 h^{-1}); DCS: distal colon condition (pH 6.8, 0.08 h^{-1}); pH var: uncontrolled pH period.

Analyses of Fermentation Products. The changes in SCFA, BCFA (isovalerate and isobutyrate), and lactate concentrations during continuous fermentation are shown in Fig. 3. Acetate was the predominant SCFA produced throughout the fermentation, while no valerate was ever detected. BCFA were present only at low concentrations (≤10 mM), which did not change for the different culture conditions tested (Fig. 3A). Acetate production was highest at the end of the initial batch fermentation and during PCS (Fig. 3B). This resulted in a significantly higher proportion of acetate during PCS compared with TCS and DCS (respectively 65.1 ± 1.2 vs 50.1 ± 0.9 and 57.0 ± 1.4%, $P < 0.0001$) (Table 4). During TCS, acetate production decreased while prop-

Table 4. Concentrations and molar proportions of different organic acids produced by fecal bacteria during continuous culture

	Fermentation conditions			*SEM*	*P*
Sampling days	*PCS* 35–38	*TCS* 45–48	*DCS* 51–54		
Metabolites[a] (mM)	117.5	111.7	113.5	1.8	0.4
Molar proportions					
Acetate (%)	65.1a	50.1b	57.0c	1.9	0.0001
Propionate (%)	12.3a	37.8b	27.7c	3.2	0.0001
Butyrate (%)	5.1a	5.0a	8.2b	0.6	0.008
Valerate (%)	nd[b]	nd	nd	nd	—
BCFA (%)	6.6	5.6	6.5	0.2	0.07
Lactate (%)	10.9b	1.5a	0.6a	1.5	0.0001

Reported data are means for four samples harvested daily during pseudo-steady states.
Values with different letters in a row differ significantly ($P < 0.05$).
[a]Defined as the sum of acetate, propionate, butyrate, valerate, BCFA (isovalerate and isobutyrate), and lactate concentrations;
[b]nd: not detected.

ionate concentration increased, amounting to 37.8% of the analyzed metabolites (Table 4). The lactate proportion significantly decreased when the pH and retention time increased, i.e., from PCS to TCS and DCS, representing 10.9, 1.5, and 0.6% of metabolites, respectively. The butyrate level was not significantly changed by the different conditions.

Discussion

In vitro models appear to be useful for studying the major interacting bacterial populations of the gastrointestinal tract, which suggests a basis for developing functional food components [18, 19]. All *in vitro* approaches currently used to investigate human large intestinal microbiota use free-cell culture fermentations [19]. Our study investigated the effects of cell immobilization on bacterial composition and activity of infant fecal microbiota in continuous cultures at different pH and feed rates approximating conditions encountered in the different colonic segments (proximal, transverse, and distal).

Because few published data are available on infant ileal chyme composition, the physicochemical environment of the infant colon or variations in substrate availability, culture dilution rates, and pH were estimated or adapted from the adult [34]. The amount of mucin added to the culture medium was that suggested by Macfarlane et al. [34], and the total transit time (corresponding to the reciprocal of dilution rate in our study) is believed to be in the range of 5.4–36.5 h, with a median value of 17.4 h in infants aged 113 days [51]. We chose for our model a retention time of 12.5 h to simulate the cecocolonic transit. The retention times in the proximal, transverse, and distal colon were adapted from adults [8] and fixed at 4, 8, and 12.5 h with dilution rates of 0.25, 0.125, and 0.08 h^{-1}, respectively. Because formula-fed infants show a fecal pH close to that of adults [17], the medium pH was successively maintained at 5.7, 6.2, and 6.8 for PCS, TCS, and DCS, respectively, as observed by Cummings et al. [7] in the adult colon. *In vivo*, proximal, transverse, and distal contents have a different composition. However, because of a lack of information about distal and transverse colon contents in infant, the same culture medium was used to simulate the three different colon segments, and consequently the proximal colon was the best simulated in terms of fermentation medium composition.

The immobilization procedure and gel composition used in the present study allowed the recovery of the dominant cultivable bacterial populations of the infant feces (Table 2). The gel beads proved to be highly resistant to mechanical and chemical degradation during long-term cultures. Gellan gum was selected from previous work on gel rheological behavior, because it provides a good entrapment matrix for temperature-sensitive bacteria such as bifidobacteria [4, 13] and suitable mechanical properties for long-term stability during continuous cultures with immobilized cells [4]. Gellan gum is a nontoxic anionic polysaccharide stabilized by a variety of monovalent and divalent cations, which are present in culture broths. Gellan also exhibits a useful synergism with other polymers, such as xanthan [25, 55]. We used a mixture of gellan and xanthan to increase strength and decrease brittleness in gels, which are two important characteristics of bead stability in bioreactors [1]. Gel beads showed good resistance to chelating agents, cell growth, and bacterial metabolism; they kept their physical integrity throughout the experiment (54 days). Moreover, they were easily dissolved in EDTA for microbiological analyses, with no apparent effects on the viability of the different populations. Despite the disappearance of part of the bacterial populations during the immobilization procedure, the bacterial diversity present in the inoculum was preserved during subsequent continuous cultures, at least at the genus level. All bacterial groups tested decreased by the same order of magnitude during immobilization, except *Clostridium* spp. and the *Bacteroides fragilis* group, which were more sensitive. The survival rates of oxygen-sensitive populations such as bacteroides and clostridia could probably be further increased by carrying out immobilization in strict anaerobic conditions, such as in an anaerobic chamber.

Continuous cultures of immobilized fecal microbiota produced extensive bacterial colonization and high population stability in gel beads throughout the fermentation process and maintained the global bacterial diversity of the inoculum. After 2 weeks of culture, the gel beads were highly colonized by the nine groups of marker bacteria (Table 2.) All simulated colonic conditions allowed cell growth and produced high and stable populations in beads, with total anaerobes reaching numbers (10^{10} CFU/g beads) comparable to infant fecal microbiota [41, 52], even during the period of uncontrolled pH (Table 2). A stable equilibrium between the bacterial groups was maintained throughout the fermentation with low variations in immobilized bacteria numbers. It has previously been shown that immobilized bacteria preferentially grow in the peripheral layer of gel beads, because of substrate, metabolic product, and possibly physicochemical (O_2, pH) gradients [5, 29]. At steady state, a biofilm of fixed density is formed, and newly formed immobilized biomass is balanced by shedding free cells into the bulk medium, contributing to system stability [30]. When culture parameters vary, immobilized cell growth rate changes, resulting in a rapid change in free cells and a continuous inoculation of the effluent, but only small changes in immobilized cell populations.

As observed for the immobilized populations, the culture effluent was also characterized by bacterial di-

versity and high viable cell numbers, comparable with those observed in the complex model of Minekus et al. [40]. In this model, the removal of metabolites and water was combined with peristaltic mixing to obtain physiological concentrations of microorganisms, dry matter, and microbial metabolites. In our study, the relatively low bacterial concentrations, approximately 10 log CFU/mL in the fecal inoculum and experimental samples, might be explained by two possible factors; (1) dilution of bacteria for the enumeration was not carried out in an anaerobic chamber; and (2) the Wilkins-Chalgren medium underestimated the total bacterial concentration. Indeed, in a recent experiment, we tested the bacterial concentration by DAPI staining followed by microscopic enumeration. A bacterial concentration of 10.8 log CFU/mL using DAPI was observed compared with only 10 log CFU/mL using the Wilkins-Chalgren medium for the same sample. Therefore, it can be assumed that a concentration of 10 log CFU/mL obtained in this study in feces, beads, and culture effluent is indeed underestimated by the Wilkins-Chalgren plate-counting method and could correspond to concentrations close to 11 log CFU/mL, which are representative of total bacterial concentrations of feces or colon. Although planktonic subpopulations were highly affected by both culture pH and dilution rates, the total bacterial concentration was very stable and no population disappeared or dominated. The period of uncontrolled pH showed that most bacterial populations were highly sensitive to pH variations at a fixed dilution rate (Figs. 1 and 2; days 19–27). The growth of *Bifidobacterium* spp. was stimulated by an acidic pH and inhibited by a basic pH, whereas the reverse was true for coliforms and staphylococci. Such pH sensitivity has already been observed for lactic acid bacteria [38], lactobacilli, streptococci, and coliforms [53] in liquid chemostats with fecal material. However, microbiological analyses of beads (Table 2) did not show a large effect of the pH-variation period on the bacterial populations, at least at the genus level. In addition, the population and metabolic product concentrations (Fig. 3) measured at the end of the stabilization period (pH 6.2) and for day 26 (pH 6.2, end of pH-variation period) were similar. Moreover, after the pH electrode was changed, the system was operated under the conditions for PCS for 10 days to reach a pseudo-steady state for the system. Therefore, we expect that the pH-variation period did not induce a large change in the composition and activity of the microbiota in the intestinal reactor.

In agreement with the effect of pH on the different populations discussed above, we observed a decrease in *Lactobacillus* spp. concomitant with an increase in coliforms in the change from PCS to DCS (Fig. 2). However, when pH was adjusted to 5.7 with a dilution rate of 0.125 h^{-1} (PCS), *Bifidobacterium* spp., with an optimum growth pH of 6.7–7 [47], was surprisingly the only population to be stimulated, rather than the acidophilic populations (*Lactobacillus* spp. and streptococci). Because bacterial growth is mediated by both pH and substrate availability, we might presume that *Bifidobacterium* spp. were more competitive for the available substrates than the acidophilic populations. It has been shown that bifidobacterial growth is stimulated by whey proteins [42], which were abundant and hydrolyzed in our culture medium. Moreover, Gibson and Wang [20] showed that compared with lactobacilli, bifidobacterial growth is strongly stimulated (2 log) by increasing the dilution rate (0.08 to 0.3 h^{-1}) irrespective of culture pH and substrate concentration. Bifidobacteria and bacteroides are the principal polysaccharides degraders in the human colon [46], and both ferment soluble starch [50], which was the main polysaccharide (6.8 g/L) in our culture medium. In contrast with bifidobacteria, which strongly decreased during TCS, the increase in bacteroides concentration might be related to their ability to compete more effectively at low growth rates under conditions of limiting substrate availability (Fig. 1) [24].

The microbiota fermentative capacity was stable from PCS to DCS with no significant difference in the sum of main metabolites concentrations (Table 4). However, the SCFA molar proportions were greatly altered by changes in pH and dilution rate, as were the planktonic bacteria. Compared to PCS, acetate proportion decreased during TCS and increased during DCS, whereas the reverse was true for propionate. Acetate production was positively correlated with bifidobacteria concentration ($r = 0.76$, $P < 0.05$) during the three successive treatments. These data suggest that acetate may be mainly produced by the bifidus pathway, which theoretically yields two lactate and three acetate from two hexose molecules [11]. The fact that lactate proportion decreased during TCS and remained low does not necessarily indicate that lactate was not produced, because other bacteria can metabolize it into SCFA [3]. Lactate production is also associated with elevated carbohydrate availability because it is typical of rapidly depolymerized substrates [32]. Its production during PCS was then in accordance with both elevated bifidobacteria numbers and high carbohydrate availability.

Compared to a three-stage continuous chemostat, each stage simulating a different section of the colon, the single-stage chemostat used in this study did not reproduce the spatial distribution and increase of N-substrates as the principal carbon source for TCS and DCS; PCS was thus likely to be better reproduced. The community structure developed during the PCS globally reflected the relative proportions of the bacterial groups and metabolites generally described in infant feces [17, 22, 39, 41] and conventional *in vitro* models [9]. During PCS, acetate (65%) was the major metabolite produced, followed by propionate (12.3%) and lactate (10.9%), while buty-

rate (5.1%) and BCFA (6.6%) were produced in smaller quantities. High proportions of lactate have already been reported *in vivo* in formula-fed infants [41]. As previously observed in infant feces [9, 39], no valeric acid was detected in our system. However, we obtained lower acetate and higher propionate ratios than in feces [17], which has already been described in conventional *in vitro* systems [34] and explained by the absence of SCFA absorption. We compared our data with infant fecal microbiota because there are no available data for bacterial composition in the infant proximal colon. Since we did not characterize the microbiota at a species level, it is impossible to state that the bacterial diversity was preserved at that level. Indeed, it is now well known that the classical culture-based methods can give a biased view of the composition of a complex microbial ecosystem, such as the intestinal microbiota [37]. However, this experiment was a first step in the characterization of this new system, in which the main challenge was to successfully immobilize and preserve complex microbiota during long-term fermentation. A complete characterization of this system with advanced molecular detection methods is currently under way in our laboratory.

To our knowledge, this work is the first reported study on the immobilization of complex fecal microbiota. The main advantage of bacterial immobilization was stability over long experimental trials, due to the continuous inoculation of the medium by shedding of free cells from highly colonized beads retained in the reactor. This ability allowed the system to rapidly restore previous equilibrium. Compared with fermentation models using free cells, the use of immobilized cells for modeling colonic fermentation presented other distinct advantages, including high cell density and the possibility of using precolonized beads with the same microbiota for testing different parameters in the same or in several experiments. Moreover, because it is known that bacterial metabolism is modified in biofilms [35], immobilization of the microbiota might then improve the colon modelization, by modifying bacterial physiology. This aspect will be also investigated in order to better characterize this model. We are also working on ways to improve the modeling of transverse and distal colon fermentations by using a three-stage chemostat with immobilized cells.

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