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Fermentation by the Human Large Intestine Microbial Community in an In Vitro Semicontinuous Culture System

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A semicontinuous culture of the microbial community of the human large intestine that was maintained over 81 days is described. The initial inoculum was feces, and about 200 ml of nutrient suspension was fed to 500 ml of fermentor contents once or twice daily. The nutrient suspension contained comminuted fibrous food, sodium deoxycholate, urea, acid-hydrolyzed casein, vitamins, and salts. The fermentation was monitored, and the major products were acetate, propionate, butyrate, methane, hydrogen, and carbon dioxide. The concentration of anaerobic bacteria was 2×10^9 per ml of culture contents and was 100 times that of fecal coliforms. When the nutrient suspension contained lettuce, celery, carrots, and unsweetened applesauce, the predominant nonsporeforming anaerobes isolated were *Bacteroides* species. When carrots and applesauce were omitted, the predominant nonsporeforming isolates were *Fusobacterium* species. On both diets, clostridia were isolated that resembled *Clostridium clostridiiforme*. The fermentation and bacteriological analyses indicated that the in vitro ecosystem appears to be a reasonable facsimile of the large intestine ecosystem.

Although activities of the normal microbial community of the human large intestine may affect health (10), the relationships between diet and human physiology on the composition and activities of the community are not clearly understood. Technical difficulties are encountered when human subjects are used to examine these interrelationships. Even when genetic and physiological variability can be discounted and diet and environment can be controlled, it is extremely difficult to measure the in vivo activities of the microbial community. Such measurements are difficult enough to do with ruminants, where it is relatively easy to control diet and environment and there is easy access to the site of microbial activity through the use of appropriate fistulae.

Several years ago, Wolin and his colleagues were able to maintain a reasonable facsimile of the rumen microbial community in vitro in semicontinuous culture (17, 20). These cultures were started with rumen contents and provided with substrates approximating normal ruminant diets. Fluid turnover was accomplished by the addition of water and artificial saliva. Except for washout of large ciliate protozoa, the microbial populations and amounts and proportions of fermentation products (short-chain volatile fatty acids [VFAs], methane, and carbon dioxide) were similar to those observed in vivo. The system was used to study features of the overall rumen fermentation such as the influence of diet, pH, and the effect of inhibitors of methanogenesis (18, 19, 21).

This report describes the development of a semicontinuous system to maintain the human large intestine microbial community in vitro. Analysis of the products and enumeration and isolation of bacteria indicated that these fermentations were similar to those of the human large intestine.

MATERIALS AND METHODS

Apparatus. A glass vessel was used to maintain 500 ml of magnetically stirred culture at 37°C (Fig. 1). The vessel contained liquid and gas sampling ports constructed from disposable plastic hypodermic syringes equipped with two-way female luer stopcocks (Popper and Sons, New Hyde Park, N.Y.). The system was initially flushed with nitrogen and closed to maintain anaerobiosis. Two football bladders, fitted with double-end shutoff Quick-connects (Crawford Fitting Co., Solon, Ohio), were used to prevent pressure buildup and acted as a ballast to eliminate negative pressure when liquid effluent was removed. To minimize corrosion of the Quick-connects, the fermentation gases were stripped of H_2S by passage through two 300-ml solutions of 5% lead acetate before collection in the football bladders. A T-line equipped with a one-way valve connected the bladders to the growth vessel and allowed gas to flow back to the growth vessel. Effluent was collected in a separatory funnel containing a saturated solution of mercuric chloride to stop fermentation when culture contents were pumped from the vessel. The sterile nutrient solution was magnetically stirred in a water-jacketed glass vessel cooled to 4°C.

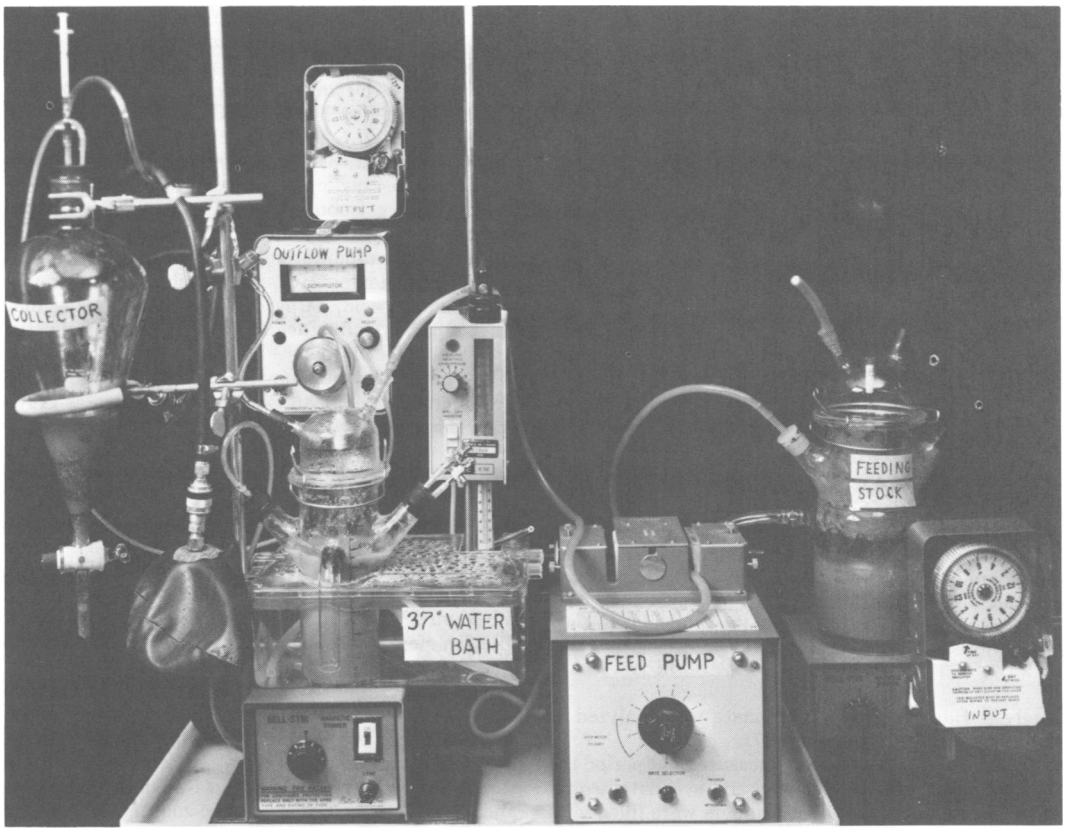

FIG. 1. *In vitro, semicontinuous fermentation system.*

The effluent and input peristaltic pumps were controlled by 24-h timers that could turn circuits on and off at 15-min intervals or multiples thereof (Intermatic Inc., Spring Grove, Ill.).

Nutrient suspension. Table 1 gives the final composition of the nutrient suspension. The lettuce, celery, and carrots were comminuted in a food processor and combined with the unsweetened applesauce. Tap water was added to 1.4 liters; the suspension was then mixed and centrifuged for 1 h at 4,080 × *g*. The food pellet was suspended in solution B and autoclaved for 1 h. The other components listed in Table 1 were combined in a single solution, filter sterilized, and added to the autoclaved food suspension. The final pH was 7.0 to 7.2. The dry weight of insoluble matter in all food suspensions ranged from 10 to 15 mg/ml.

The nutrient suspension was checked daily for sterility by microscopic examination and by inoculation of nutrient broth and incubation at 37°C. If contamination was noted, the suspension was replaced. Occasional contamination occurred but was eliminated before significant amounts of material were pumped into the fermentor.

Inoculum. The fermentation was started with 156 g (wet weight) of freshly voided feces from a healthy adult male on a typical American diet. The feces were mixed with 200 ml of solution B (Table 1) and filtered through a double layer of cheesecloth. The fecal suspension was added to the culture vessel and brought to 500 ml with solution B. After the apparatus was gassed with N_2, the system was continuously stirred and incubated for 18 h before initiation of nutrient addition from the nutrient reservoir.

Feeding regimen. Initially, the culture was fed at 12-h intervals. Before feeding, approximately 200 ml of culture contents was removed, and 200 ml of nutrient suspension was then added to the culture.

Fermentation analyses. During each experimental period, samples (3 to 5 ml) were taken directly from the culture vessel before initiation of the morning removal/feeding cycle. Samples for VFA analysis were acidified with 0.2 ml of 2 M formic acid per ml, centrifuged for 5 min at 3,015 × *g*, and frozen. The acidified samples were rapidly thawed and analyzed for acetate, propionate, and butyrate by gas-liquid chromatography as described by Chen and Wolin (7).

The remaining portion of the daily sample was centrifuged for 5 min at 3,015 × *g* and frozen for later enzymatic or chemical analyses. Formate was measured by the formyltetrahydrofolate synthetase method of Rabinowitz and Pricer (16). The synthetase was a gift from J. C. Rabinowitz. Ethanol was assayed with alcohol dehydrogenase (4). Lactate was chemically assayed by the Barker-Summerson method (2). Lactate isomers were determined enzymatically (Boehringer Mannheim Corp., Methods of Enzymatic Food

TABLE 1. *Composition of nutrient suspension*

Component	Amt	
	Per liter	% (wt/vol)
Addition		
Lettuce	100 g, wet	10
Celery	100 g, wet	10
Carrots	100 g, wet	10
Unsweetened applesauce	100 g, wet	10
Solution A		
NaCl	2.8 g	0.28
Sodium deoxycholate	0.35 g	0.035
K_2HPO_4	0.35 g	0.035
Solution B		
$NaHCO_3$	11 g	1.1
Urea	0.5 g	0.05
Hemin	50 μg	0.00005
Casein[a]	2 g	0.2
Vitamin mixture[b]	5 ml	0.5 (vol/vol)

[a] Acid hydrolyzed, vitamin-free.

[b] Contained 20 mg each of thiamine-hydrochloride, nicotinamide, riboflavin, pyridoxine hydrochloride, and calcium D-pantothenate, 2 mg of cyanocobalamin, 10 mg of biotin, 1 mg of *p*-aminobenzoic acid, and 0.5 mg of folic acid per 100 ml.

Analysis Bulletin 77/78). Succinate was analyzed as described by Chen and Wolin (7).

On the 1st day of the 4-day measurement period the football bladders were removed and evacuated to remove accumulated gas. The bladders were filled with a total of 500 ml of N_2 and reattached to the apparatus before the start of the morning feeding cycle. On the 4th day, the bags were removed before the morning cycle. A matching Quick-connect, fitted with a septum, was attached to the bags, and samples were removed with a syringe for gas analysis. Methane and H_2 were determined by gas chromatography as described by Chen and Wolin (6). The total gas accumulated during the 72-h period was determined by attaching each bag to an inverted bottle fitted with a matching Quick-connect and filled with a 0.5% citric acid and 20% NaCl solution. The amount of liquid displaced when the bags were emptied was measured and used to calculate the total CH_4 and H_2 accumulated during 3 days.

The volume of effluent removed each day for 3 days was used to calculate the average daily accumulation of soluble and gaseous fermentation products.

Diurnal product formation. After the removal of fermentor contents in the morning, a zero-time sample was taken 5 min before the nutrient suspension was fed. Samples were then removed at intervals for 12 h, and all samples were analyzed for acetate, propionate, butyrate, and formate. All values were corrected for dilution of the zero-time values caused by the feeding of the nutrient suspension.

Anaerobic media. Procedures for dispensing media, autoclaving, and culture transfer were as previously described (14). For routine transfer and fermentation analyses, media were in 5-ml amounts in serum bottles with about 4 ml of gas space. All incubations were at 37°C. The complex basal medium (SMS) contained per liter: Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 5 g; yeast extract (Difco Laboratories, Detroit, Mich.), 2 g; K_2HPO_4 and KH_2PO_4, 0.24 g each; $(NH_4)_2SO_4$, 1.24 g; NaCl, 0.48 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $CaCl_2 \cdot 2H_2O$, 60 mg; $FeSO_4 \cdot 7H_2O$, 4 mg; Na_2CO_3, 4.0 g; resazurin, 1 mg; dithioerythritol, 15.4 mg; cysteine $HCl \cdot H_2O$, 0.875 g; $Na_2S \cdot 10H_2O$, 0.375 g; isobutyric, 2-methylbutyric, *n*-valeric, and isovaleric acids, 0.1 ml each; hemin, 2 mg; thiamin·HCl, calcium D-pantothenate, nicotinamide, riboflavin, and pyridoxine·HCl, 2 mg each; biotin, 1 mg; *p*-aminobenzoic acid, 100 μg; cyanocobalamin, 20 μg; folic acid, 50 μg; and folinic acid, 10 μg. The pH was adjusted to 7.0 with NaOH before boiling. After rapid cooling in icy water, the medium was gassed with 100% CO_2. Carbonate, cysteine, and sulfide were added as previously described (14).

Microbiological analyses. During each 4-day measurement period, portions of the fermentor contents were removed before the morning addition of feedstock. One milliliter was syringed into a serum bottle containing 9 ml of anaerobic dilution solution (5) and about 20 glass beads (about 5 mm in diameter). The bottle was swirled for 3 min in icy water and mixed on a Vortex mixer for 2 min just before the next dilution step. Subsequent dilutions were mixed for 15 s.

The anaerobic and facultative anaerobic bacteria were enumerated in SMS medium with 0.1% each glucose, cellobiose, and maltose and 2% Difco agar. Ten-fold serial dilutions were performed, and duplicate roll tubes were made with 0.1 ml of the 10^{-6}, 10^{-7}, and 10^{-8} dilutions. Colonies were counted at 48 h and 5 days.

Fecal coliforms were enumerated by the membrane filter method, and bacteria were enumerated on standard plate count medium (1).

Isolation of predominant anaerobes. Colonies were picked from the highest dilution and subcultured in SMS with 0.5% glucose or SMS with 0.1% glucose, maltose, and cellobiose. Subcultures were restreaked on SMS agar with 0.1% glucose, maltose, and cellobiose. Cell morphology was examined by phase-contrast microscopy and Gram stain.

Substrate utilization. SMS medium was used to determine the ability of anaerobic isolates to use the following substrates: 1% glucose, fructose, L(+) and D(−)-arabinose, mannitol, sucrose, xylose, melezitose, mannose, lactose, and L-malate (monosodium); 0.6% DL-sodium lactate; 0.5% threonine; and 0.2% starch. Cellulolytic activity was examined in SMS containing a strip (5 by 28 mm) of Whatman no. 1 filter paper. Esculin hydrolysis was determined with 0.5% esculin. Indole reaction was determined with 0.5% glucose and 2.5% Trypticase. The requirement for VFAs was examined with 0.5% glucose in SMS from which the VFAs were omitted. Bile sensitivity was examined with 0.5% glucose, 2% dehydrated oxygall, and 0.1% sodium deoxycholate. The turbidity of cultures in 5-ml serum bottles was measured at 660 nm in a Bausch & Lomb Spectronic 70 spectrophotometer against an uninoculated blank of the same medium. The path length was about 2 cm.

Fermentation products. Isolates were grown for 24 h on 0.5% glucose in SMS. After incubation, gas production and hydrogen were determined as described previously (6, 14). Cultures were centrifuged at 18,000 × *g* for 10 min at 0°C. The clear supernatants were either analyzed immediately or frozen at −20°C for later analyses using the procedures described for the semicontinuous culture system.

RESULTS

Measurements of products were initiated after 29 days of operation of the fermentation unit with twice-a-day feeding of the complete nutrient suspension (Table 1). VFAs were determined each day for 4 days, and the cumulative CH_4 and H_2 production was determined for the entire 4-day period. The results indicated that an apparent steady state had been reached because there were only small daily variations in the amounts of VFAs produced (Table 2). Acetate was the major VFA product. Acetate carbon was 69% of the total carbon recovered in the VFAs, and butyrate, propionate, and formate carbon were 24, 6, and 1%, respectively. Approximately 49 ml of CH_4 and 146 ml of H_2 were formed during the 4-day measurement period (1.6 mmol of CH_4 and 4.6 mmol of H_2 per liter; Table 2). If all of the CH_4 produced was derived from the reduction of CO_2 by H_2, the H_2 produced and used for methane formation was 196 ml ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$).

Decrease in feeding rate. Nutrient feeding was decreased to once a day on the 32nd day, and two separate 4-day measurement periods were used to monitor the fermentation (days 37 to 40 and 43 to 46). VFAs were again analyzed each day of the 4-day period, and total CH_4 and H_2 were measured for the 4-day period. Since there were no major differences in the results for the two measurement periods, all values were averaged. The decrease in the number of feedings per day appeared to cause an increase in the production of propionate and methane and a decrease in the production of butyrate and hydrogen (Table 2). Feeding rates were 191 ml per 500 ml of fermentor contents per day as compared with 460 ml per 500 ml of fermentor contents per day in the twice-a-day feeding experiment. Recovery in acetate, butyrate, and propionate was 66, 17, and 17%, respectively, of the total carbon in the VFAs. Acetate and total VFA production per liter was approximately the same as that obtained with the twice-a-day feeding schedule. Approximately 140 ml of CH_4 (the equivalent of 560 ml of H_2) and 6 ml of H_2 were formed during the 4-day period.

Omission of carrots and applesauce. The composition of the feedstock was changed by omitting applesauce and carrots and increasing the amount of lettuce and celery added to the nutrient suspension from 100 to 200 g of each constituent per liter. Feeding of the new suspension commenced on day 54, and products were measured, as described previously, on days 64 through 67. Total VFA production decreased from 91.2 to 46.3 mmol/liter, but the percentage distribution of VFAs was approximately the same as in the previous feeding period (Table 2). Methane production increased from 10.9 to 18.0 mmol/liter when the new nutrient suspension was used (Table 2). Total methane formed was 249 ml (the equivalent of 996 ml of H_2), and 8 ml of H_2 was produced.

Omission of casein hydrolysate. We omitted hydrolysate from the nutrient suspension on day 72 and measured fermentation products, as described previously, on days 78 to 81. The amounts of kinds of VFAs were similar to those obtained with casein hydrolysate, but there was a significant decrease in the production of CH_4,

TABLE 2. *Fermentation products of the semicontinuous culture system*

Days	Fiber[a]	Amt produced[b]									
		Acetate		Propionate		Butyrate		Formate		CH_4	H_2
		mmol/liter	%[c]	mmol/liter	%	mmol/liter	%	mmol/liter	%		
29–32	LeCeCaAp, 230 ml/12 h	71.6 (1.2)	77	4.4 (1.8)	4	12.4 (7.8)	17	1.8 (0.8)	2	1.6	4.6
37–40 and 43–46	LeCeCaAp, 191 ml/24 h	70.1 (7.6)[d]	76	12.2 (3.1)[d]	13	8.8 (0.9)[d]	10	1.0 (0.2)[d]	1	10.9 (3.9)[e]	0.5 (0.2)[e]
64–67	LeCe, 206 ml/24 h	35.1 (3.7)	76	5.4 (0.8)	12	5.8 (0.2)	12	0.0	0	18.0	0.6
78–81	LeCe − casein, 232 ml/24 h	36.4 (1.3)	68	7.7 (0.5)	15	7.5 (0.4)	14	1.4 (0.1)	1.4	4.3	0.1

[a] Le, lettuce; Ce, celery; Ca, carrots; Ap, applesauce.
[b] Means of four daily measurements; numbers in parentheses are standard deviations.
[c] Percentage of total VFAs based on means.
[d] Means of eight daily determinations.
[e] Means of two 4-day total gas measurements.

with no increase in the production of H_2 (Table 2). There was some change in the proportions of VFAs produced.

Time course of VFA production. One experiment was performed to determine the time course of VFA production after feeding. This was done early in the study (day 24) when the original, complete nutrient suspension was fed twice daily. A fast rate of acetate production occurred for approximately 2 h which was followed by a slow production rate that continued for the remaining 12 h of the feeding period (Fig. 2). Butyrate was formed more slowly than acetate, but its production ceased after about 4 h. Propionate production was slow and was detectable only between 4 and 12 h. Formate was formed early in significant amounts, but its formation was accompanied by utilization. Whereas 7 mM formate was detected at 4 h, none was present after 12 h.

Viable bacteria. Concentrations of bacteria in the semicontinuous culture were determined at five measurement periods during the 81-day operation of the unit (Table 3). The means of all anaerobic, fecal coliform, and standard plate counts during the 81-day period were 2×10^9, 1.3×10^7, and 1.6×10^7 bacteria per ml of culture contents, respectively. The ratio of the means of anaerobic to standard plate counts was 100:1.

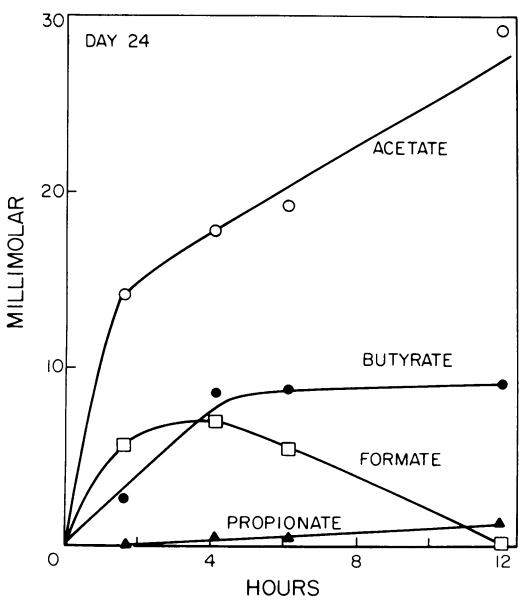

FIG. 2. *Time course of VFA production. The millimolar concentrations are the concentrations at the indicated time minus the concentration at time zero. The fermentor was on the complete nutrient suspension given in Table 1 and was fed twice daily.*

TABLE 3. *Concentrations of viable bacteria*

Days	Fiber[a]	Feedings		Log_{10}/ml		
		No./day	ml/day	Anaerobic	Fecal coliform	Standard plate count
29-32	LeCeCaAp	2	459	9.3	7.5	7.6
37-40	LeCeCaAp	1	192	9.3	7.3	7.6
43-46	LeCeCaAp	1	191	9.3	6.8	6.9
64-67	LeCe	1	206	8.9	6.8	7.0
78-81	LeCe − casein	1	232	9.3	7.1	7.4

[a] See Table 2.

The ratio of the means of the fecal coliform to standard plate counts was 0.6:1.

Isolation of nonsporeforming anaerobic bacteria. Nonsporeforming anaerobes were isolated from roll tubes that were inoculated with 0.1 ml of the 10^{-7} dilution of day 53 and day 66 culture contents. On day 54, the nutrient suspension was altered by eliminating the carrots and applesauce and increasing the concentration of lettuce and celery.

Six gram-negative, nonmotile, nonsporeforming rods were purified from the culture on day 53. Each isolate was highly pleomorphic. Short to long rods with rounded ends, occurring singly and in chains, were observed in wet mounts. For further characterization, the isolates were designated rods A, B, C, D, E, and F. They all produced acetate and propionate as products of glucose fermentation (Table 4). Only rods A, B, and E produced significant amounts of lactate, and only the D-isomer of lactate was detected. None of the isolates produced butyrate, ethanol, formate, or hydrogen.

All of the rods were indole positive, hydrolyzed esculin, and were insensitive to bile. The six isolates were similar in their ability to use a variety of substrates for growth (Table 5). However, rods E and F did not use melezitose. Based on the morphology and fermentation characteristics, the isolates from day 53 resembled those reported for *Bacteroides thetaiotaomicron* (12).

Three nonmotile, nonsporeforming rods and one nonmotile coccus were isolated from the culture on day 66. The three rods were gram negative. Two isolates, designated rod H, were identical in morphology and physiology. Short rods with slightly tapered ends occurred singly and in chains of no more than three cells. They produced acetate, butyrate, lactate, formate, and H_2 as products of glucose fermentation (Table 6). Only the L-isomer of lactate was detected. They did not produce propionate or ethanol. The rod H isolates were indole negative, hydrolyzed esculin, and were insensitive to bile. They

TABLE 4. *Glucose fermentation products of nonsporeforming anaerobic bacteria isolated on day 53*[a]

Rod	Amt produced (mM)[b]		
	Acetate	Propionate	Lactate
A	11.6	3.0	3.4
B	9.8	2.3	4.0
C	8.9	1.6	tr[c]
D	11.4	3.1	tr
E	9.9	2.1	4.2
F	28.9	7.7	0

[a] One feeding per day of 191 ml of nutrient suspension containing lettuce, celery, carrots, and applesauce.
[b] Produced from fermentation of 0.5% glucose.
[c] tr, Trace; less than 2 mM.

did not require VFA supplementation for growth.

The third rod isolated was morphologically distinct from rod H and was designated rod I. Rod I was a long, slender rod and occurred singly and in pairs. It was indole negative and hydrolyzed esculin, but growth was inhibited by bile. Rod I also required VFAs for growth. The products of glucose fermentation by rod I were similar to those of rod H except that rod I did not produce H_2 (Table 6). It also produced only the L-isomer of lactate.

The use of substrates was not extensively studied with the day 66 group of nonsporeforming rods. Rods H and I grew on glucose, fructose, and mannose but not cellulose, L-malate, DL-lactate, or threonine. Rod I grew on starch, whereas rod H did not use starch. Despite the strain differences observed between the isolates of rods I and H, their characteristics most closely resembled those reported for *Fucobacterium mortiferum* (12).

The anaerobic coccus was gram negative, nonmotile, and bile sensitive. The coccus occurred singly and in pairs. It produced acetate, ethanol, formate, and H_2 as major products of glucose fermentation (Table 6). It did not produce butyrate or propionate. The coccus was not further characterized. However, its fermentation products were similar to those of *Ruminococcus albus* and *R. bromii* (12).

Sporeforming anaerobic rods. Two isolates from the day 53 and one isolate from the day 66 samples were gram-negative, nonmotile rods that produced oval, terminal to subterminal spores. They were morphologically identical rods that occurred singly and in pairs. They fermented 0.5% glucose to about 20 mM acetate, 27 mM ethanol, 13 mM formate, and 27 mM H_2. They did not produce propionate, butyrate, or lactate.

TABLE 5. *Substrate utilization by day 53 isolates*

Substrate	Utilization by given rod[a]					
	A	B	C	D	E	F
None	—	—	—	—	—	—
Glucose	3+	3+	3+	3+	3+	3+
Fructose	3+	2+	3+	3+	2+	3+
Mannose	3+	3+	3+	3+	3+	3+
L-Arabinose	3+	2+	3+	2+	1+	3+
D-Arabinose	2+	2+	3+	3+	1+	3+
Xylose	3+	3+	3+	3+	2+	3+
Lactose	3+	3+	3+	3+	3+	3+
Sucrose	3+	3+	3+	3+	2+	3+
Melezitose	3+	2+	2+	2+	—	—
Starch	3+	3+	3+	3+	3+	3+

[a] 3+, Absorbancy at 660 nm (A_{660}) of 1.0 or higher at 24 h; 2+, A_{660} of 1.0 or higher at 48 h; 1+, A_{660} of 0.7 or higher at 72 h; —, A_{660} of 0.3 to 0.6 at 72 h. Growth on cellulose was scored negative if the filter paper remained intact for 4 weeks. All isolates were negative with mannitol, cellulose, L-malate, and DL-lactate.

TABLE 6. *Glucose fermentation products of nonsporeforming anaerobic bacteria isolated on day 66*[a]

Isolate	Amt produced (mM)[b]					
	Acetate	Butyrate	Lactate	Ethanol	Formate	H_2
Rod H[c]	4.0	13.6	5.4	0	10.4	14.3
Rod I	10.9	9.7	10.1	0	21.7	0
Coccus	18.0	0	tr[d]	19.8	9.2	22.4

[a] One feeding per day of 206 ml of lettuce-celery nutrient suspension.
[b] Produced from fermentation of 0.5% glucose.
[c] The two strains of rod H produced the same amount of products.
[d] tr, Trace; less than 2 mM.

The isolates did not hydrolyze gelatin, were esculin and indole positive, and fermented mannose but not mannitol. Although the isolates did not produce lactate, the above characteristics resemble those of *Clostridium clostridiiforme* (12).

DISCUSSION

Certain assumptions about the human large intestine ecosystem were made in designing this in vitro system. It was assumed that most microbial growth and metabolism occurs immediately after the entry of ileal fluid into the large intestine and before the removal of water from the large intestine, which concentrates the mass of microorganisms and other residues of digestion that are eventually excreted. It was also assumed that the microbial community of the fecal inoculum was representative of the large intestine microflora. The pulsed inputs of nutrients in

large volumes one or two times a day were introduced to imitate the pulsed entry of ileal fluid into the large intestine that occurs after feeding. Foods with high concentrations of insoluble carbohydrate that are thought to escape monogastric digestion were used as primary carbon and energy sources. Urea was used as a major source of nitrogen, and sodium deoxycholate was included to represent the presence of bile salts. The assumptions are doubtlessly oversimplifications of the characteristics of the in vivo system (9), and even the assumptions were compromised. For example, nutrients were pulse fed only one or two times a day, and vitamin and casein hydrolysate supplements were added without any justification based on the known characteristics of the in vivo ecosystem.

Despite the simplifying assumptions and compromises, the microbial fermentation in the in vitro system was very similar to that which is believed to occur in vivo. Short-chain VFAs were major products, and the concentrations of acetate, propionate, and butyrate were similar to those found in feces (8, 22). Formate has been reported to be present in feces, but insufficient information is available about the variation of its concentration. The diurnal study showed that formate was produced and disappeared within a 12-h period, in contrast to the other VFAs. This indicates that the amount of formate in the culture system and in feces may be highly variable and dependent on the balance between those microorganisms that produce and those that use formate. Several analyses were conducted for ethanol and lactate, but they were not detected. A few analyses were made for succinate by gas chromatographic procedures, and succinate was detected at a level of 3 to 5 mM. Further studies are necessary to clarify the significance of succinate as a fermentation intermediate or product.

The gas products of the in vitro fermentation were the same as those formed in vivo. The highest rate of methane production was about 83 ml of methane per day when the fermentor was fed 206 ml of nutrient suspension once a day (18 mmol/liter; Table 2). Methane-producing adult humans produce about 700 to 4,500 ml per day (based on pulmonary excretion rates [3]). The lower daily production rates probably are related to the lower daily nutrient input in the in vitro as compared with the in vivo system. Higher inputs per feeding were not practical with the in vitro system because it was difficult to pump more concentrated nutrient suspensions into the fermentor. It was also not possible to feed more than once per day and still maintain significant methanogenesis (Table 2). Apparently methanogens were not able to grow to high enough concentrations to produce significant methane unless the retention time was decreased by the reduced rate of feeding. Other factors, such as the presence and absence of casein hydrolysate and the nature of the carbon and energy sources, influenced the amount of methanogenesis. Further studies are necessary to provide a more complete understanding of the microbial interactions responsible for methane production. Feeding rate (retention time) also influenced the relative amounts of propionate and butyrate produced. Increased retention time led to the production of relatively more propionate (Table 2).

Although the amounts of lettuce and celery were doubled when carrots and applesauce were omitted from the feedstock, total VFA production was about one-half that obtained with the original, complete nutrient suspension. This indicates that the carrots and applesauce were contributing much more carbohydrate than equivalent amounts of celery and lettuce. We did not determine the composition of the feed mixtures after their preparation, and additional studies are necessary to evaluate product formation in terms of amounts and kinds of chemical constituents of the feedstock.

The feeding rate or nutrient composition fed to the in vitro culture did not significantly change the numbers of viable bacteria recovered in the media used for enumeration. Throughout the study, the anaerobic population was about 2×10^9 per g of culture and was 100 times higher than fecal coliforms or bacteria able to grow on standard plate count agar. The actual concentration of bacteria was probably much higher. Microscopic examination of the 10^{-1} dilution showed considerable numbers of bacteria adhering to nutrient particles. Microscopic examination indicated that mixing the 10^{-1} dilution beyond 2 min did not increase the number of bacteria dislodged from the particles. Mastromarino et al. reported 7.4×10^{10} anaerobic bacteria per g (wet weight) of feces from healthy subjects and a mean ratio of about 94:1 of anaerobic to aerobic bacteria (13). The concentration of anaerobic bacteria in the in vitro culture is lower than in feces because the culture contents are more dilute than feces. The effect of the amount of nutrients supplied on the concentration of microorganisms in the culture has not yet been investigated.

Bacteroides, Fusobacterium, Ruminococcus, and *Clostridium* species were isolated from the in vitro system and have been shown to be present in high concentration in human feces (11, 13, 15). When the in vitro unit was fed a

complete nutrient suspension, the major nonsporeforming rods were propionate producers (*Bacteroides*), whereas only butyrate producers (*Fusobacterium*) were isolated when carrots and unsweetened applesauce were omitted from the nutrient suspension. This was surprising in view of the fact that the proportions of propionate to butyrate produced during fermentation of the two nutrient suspensions in the in vitro culture did not significantly change. The methanogenic population of the in vitro system was not closely monitored. However, methanogens were observed in culture contents during periods of methane production in the unit as evidenced when the cultures were examined by fluorescence microscopy. Detailed enumeration of methanogens was not carried out in this study, but a methanogen was isolated that was characterized as *Methanobrevibacter smithii* (unpublished data).

Since the main goal of the study was the development of an in vitro fermentation system that appeared to be a reasonable facsimile of the large intestine fermentation, we did not attempt to design experiments with the purpose of carefully evaluating the relationship between microbial activities and specific nutrient inputs or system dynamics. The system appears to be capable of providing information about these relationships, and additional studies should clarify some of the relationships discussed above. The in vitro system should also be suitable for a variety of other studies of microbial activities that relate to the activities within the large intestine. The diurnal studies of VFA production illustrate that the in vitro system can be used for short-term studies of microbial metabolism as well as long-term studies of the influence of the chemical and physical environment on the characteristics of the microbial community. The bacteriological analyses of the in vitro system indicate the system's utility for studying populations as well as activities.

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