Different Substrates and Methane Producing Status Affect Short-Chain Fatty Acid Profiles Produced by In Vitro Fermentation of Human Feces[1,2]

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ABSTRACT Five different substrates, i.e., lactulose, rhamnose, cornstarch, guar and ileostomy effluent, were used to determine whether methane producing status alters the production of short-chain fatty acids (SCFA) in methane producers (MP; $n = 6$) and nonproducers (MNP; $n = 5$). Fecal samples from MP and MNP were fermented with the five substrates using an in vitro fermentation method. Subjects with a mean breath methane concentration > 0.045 μmol/L above ambient air were classified as MP. Fermentation was stopped and samples were obtained at 3, 5 and 24 h. An HPLC method was used to measure the SCFA, acetate, propionate, isobutyrate, butyrate, valerate and isocaproate. A significant interaction between methane producing status and time for acetate production from lactulose was observed. There were no differences in fermentation of the four remaining substrates between MP and MNP, but there were significant differences among substrates in the two groups combined. Acetate production from lactulose was significantly greater than from the four other substrates, whereas that from ileostomy effluent was significantly less than the four other substrates. The amount of propionate produced from rhamnose was significantly higher than from the other substrates. The amount of butyrate produced from lactulose and cornstarch was significantly higher than from the other substrates. We conclude that differences exist in the fermentation patterns of lactulose, rhamnose, cornstarch, guar and ileostomy effluent. Methane producing status may influence fermentation patterns only of substrates that are largely fermented to acetate and not others. J. Nutr. 130: 1932–1936, 2000.

KEY WORDS: • *humans* • *methane* • *short-chain fatty acids* • *in vitro fermentation*

The principal end products of colonic fermentation are short-chain fatty acids (SCFA),[4] carbon dioxide, hydrogen and methane. The last-mentioned is produced by highly anaerobic methanogenic bacteria via the reduction of carbon dioxide. Excretion of methane in the breath can be used as a simple indicator of methane production in the colon (Bond et al. 1971). All humans harbor methanogens in the colon, but pulmonary methane excretion is observed only in subjects with $>10^8$ *Methanobrevibacter smithii*/g dry feces (Miller and Wolin 1986).

A higher fasting serum acetate concentration was observed in methane producers (MP) compared with methane nonproducers (MNP) by Wolever et al. (1993), and it was suggested that this may be due to an increased rate of colonic production of acetate in producers compared with nonproducers. The mechanism for the increased rate of colonic acetate production was hypothesized to be due to the presence of methane-producing bacteria, which enhance acetate production from other species as observed by Chen and Wolin (1977) using pure cultures of rumen bacteria.

Very few human studies have looked at the pattern of colonic SCFA production in methane producers compared with nonproducers. Wolin and Miller (1983) found no significant differences in the proportion of SCFA in the feces of producers and nonproducers. Weaver et al. (1989) studied fermentation of glucose and cornstarch in a producer and nonproducer over a 3.5-y period and observed that methanogenesis was accompanied by less propionate and more acetate production. Thus, our aim was to compare SCFA profiles in methane producers and nonproducers after in vitro fecal fermentation with different types of substrate.

SUBJECTS AND METHODS

We studied 11 healthy subjects (6 MP and 5 MNP) using a protocol approved by the Human Subjects Review Committee of the University of Toronto. Subjects with a history of diabetes, a thyroid disorder, liver disease, any gastrointestinal disorders or antibiotic use in the 3 mo before the study were excluded. Subjects were classified as MP and MNP using alveolar breath samples collected in 20-mL

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[4] Abbreviations used: MNP, methane nonproducer; MP, methane producer; PR:AC, propionate:acetate ratio; SCFA, short-chain fatty acids.

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syringes by the subjects after fasting and at hourly intervals for 4 h using a modified Haldane-Priestly tube (Metz et al. 1976). Before collection, subjects were asked to breathe normally to prevent precollection hyperventilation. Breath hydrogen and methane were measured against known standards by gas chromatography (Quintron Microlyser, Model DP, Milwaukee, WI) on the same day the sample was collected. Subjects also collected a sample of room air in a 20-mL syringe to measure breath methane and hydrogen concentrations in room air. Breath methane concentration was determined by subtracting the methane of room air from the average concentration in the samples collected. Subjects whose mean corrected breath methane concentrations were <0.045 μmol/L above ambient air were considered MNP. MP were defined as subjects with a corrected breath methane concentration > 0.045 μmol/L.

Fresh fecal samples, collected individually from subjects who had been consuming their regular diet, were used as an innoculum source. A batch-culture fermentation technique (McBurney and Thompson 1987) was used, and the fecal samples were fermented with five different types of substrate, lactulose (4-*O*-β-D-galactopyranosyl D-fructofuranose), α-L-rhamnose (6-deoxy-L-mannose), gum guar and cornstarch, which were obtained from Sigma Chemical (St. Louis, MO) and freeze-dried ileostomy effluent. On analysis, ileostomy effluent on a freeze-dried basis was composed of 56% carbohydrate, 21.8% protein, 2.3% fat, 35% fiber, 20.8% available carbohydrate, 5.8% moisture and 14.4% ash. Replicates were analyzed for each substrate at each time. Fermentation was stopped and samples were collected at 0 h and after 3, 5 and 24 h of fermentation. The fermentation samples were stored at −70°C until analysis.

The fermentation samples were prepared using a method modified from Chen and Lifschitz (1989). The frozen fermentation samples were thawed and ~15 mL was centrifuged at 9000 × *g* at 4°C for 20 min. The supernatant was filtered through a Millex-GS 0.22-μm filter unit (Millipore, Bedford, MA) attached to a syringe to remove bacterial cells. Another filtration was then done through a microconcentrator (centricon-3, Amicon, Danvers, MA) with a molecular-mass cutoff of 3000 Da, by centrifugation (7000 × *g* at 4°C for 1 h). The filtrate was then analyzed using a HPLC method for the SCFA, acetic, propionic, isobutyric, butyric, valeric and isocaproic. Ethylbutyric acid was used as an internal standard. The HPLC system consisted of a Waters Model 510 HPLC Pump (Waters Division of Millipore, Milford, MA), Aminex HPX-87H Ion Exclusion Column (7.8 mm i.d. × 30 cm, Biorad, Richmond, CA), a Micro-Guard Cation-H guard column (4.6 mm i.d. × 30 mm, Biorad) and a HPLC column heater (Biorad) and a Model 787A Variable Wavelength detector (Micromeritics Instrument, Norcross, GA), which was set at a wavelength of 210 nm and a sensitivity of 2.56 AUFS. The mobile phase was 0.005 mol/L sulfuric acid (Fisher Scientific, Nepean, ON, 95.5–96.5% pure) filtered, degassed and pumped at a flow rate of 0.7 mL/min through the column heated to 60°C. Quantification of the peaks by height was achieved by a HP 3396 Series II Integrator (Hewlett-Packard, Avondale, PA) and CPS 100 software (A.I.M. Instruments, Calgary, Canada) using the internal standard method.

SCFA amounts are expressed as mmol/g carbohydrate (mmol/g CHO). We assumed that lactulose, rhamnose and cornstarch were 100% carbohydrate; from published values, guar was 89.4% carbohydrate (McBurney and Thompson, 1989) and carbohydrate in freeze-dried ileostomy effluent was calculated to be 56%.

Statistical analysis was done using Systat for Windows Version 5 (Systat, Evanston, IL). The main end point was SCFA at 24 h. For SCFA at 24 h, significance was assessed by ANOVA using the General Linear Model and testing for the effects of group (i.e., methane producer status), substrate and group × substrate interactions. Within substrates, a similar ANOVA was performed testing for group, time and time × group interactions. For comparing individual means, the Newman-Kuels procedure was used to adjust for multiple comparisons. Unpaired Student's *t* test using a computer spreadsheet in Microsoft Excel 97 (Microsoft, Seattle, WA) was used to analyze the data in **Table 1**. Results are expressed as means ± SEM. Differences were considered significant at $P \leq 0.05$.

RESULTS

We studied 6 MP (3 men and 3 women) and 5 MNP (2 men and 3 women). There were no differences between MP and MNP in age or body mass index (Table 1).

There were no significant differences in production of acetate, propionate, isobutyrate, butyrate, valerate and isocaproate from lactulose, rhamnose, cornstarch, guar and ileostomy effluent at 3, 5 and 24 h between MP and MNP (**Fig. 1**). However, there was a significant interaction between methane producing status and time for production of acetate from lactulose ($P = 0.01$); at 5 h, MNP produced significantly more acetate compared with MP, whereas at 24 h, MNP produced significantly less acetate than MP. The amount of individual SCFA produced in vitro in MP and MNP as a percentage of total SCFA did not differ.

Lactulose fermented in vitro at 24 h produced significantly increased concentrations of acetate compared with the other substrates ($P < 0.01$) (**Fig. 2**A). Rhamnose produced significantly more propionate compared with the other substrates ($P < 0.001$) (Fig. 2A). Significantly increased concentrations of butyrate were produced by lactulose and cornstarch at 24 h compared with the other substrates ($P < 0.001$) (Fig. 2A).

Because isobutyrate, valerate and isocaproate were produced in small amounts, they were combined for statistical purposes. There was no significant difference in the total amounts of these branched-chain acids produced at 24 h among the substrates. As a percentage of total SCFA produced at 24 h, ileostomy effluent produced significantly greater amounts of the branched-chain acids compared with the other substrates ($P < 0.01$) (Fig. 2B).

Total SCFA (i.e., acetate, propionate, butyrate, isobutyrate, valerate and isocaproate) production from lactulose and guar were significantly higher compared with cornstarch and ileostomy effluent at 24 h in the groups combined ($P < 0.05$) (Fig. 2C).

The PR:AC (propionate:acetate) ratio at 24 h was significantly higher with rhamnose compared with lactulose, rhamnose, cornstarch, guar and ileostomy effluent in the group combined.

TABLE 1

Age, body mass index (BMI), and breath hydrogen and methane in methane producers (MP) and methane nonproducers (MNP)[1]

	n (M:F)	Age	BMI	Hydrogen	Methane
		y	*kg/m²*	*μmol/L*	
MP	3:3	30.0 ± 2.8	22.9 ± 1.1	0.23 ± 0.08	0.90 ± 0.32
MNP	2:3	29.0 ± 2.1	23.8 ± 1.0	0.50 ± 0.15	<0.05

[1] Values are means ± SEM; M, male; F, female.

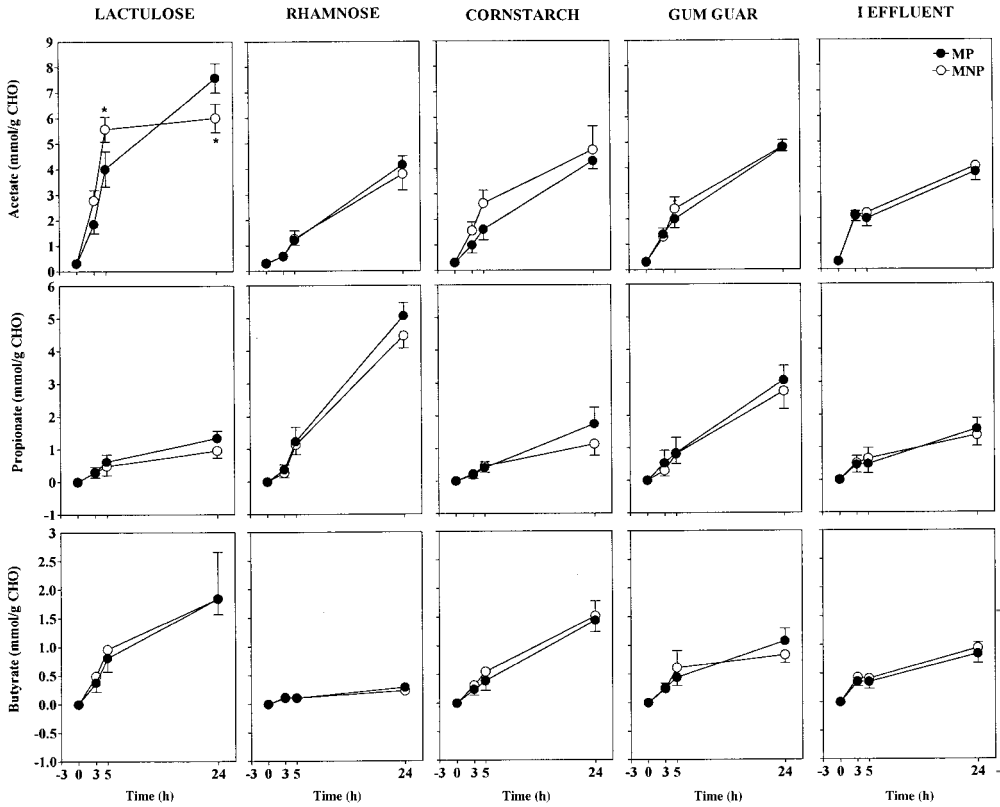

FIGURE 1 Acetate, propionate and butyrate produced at 3, 5 and 24 h from lactulose, rhamnose, cornstarch, gum guar and ileostomy effluent (I Effluent) in feces of methane producers (MP) and nonproducers (MNP). Values are means ± SEM, $n = 6$ for MP and $n = 5$ for MNP; CHO, carbohydrate. *Significant interaction between methane producing status × time, $P = 0.01$.

DISCUSSION

This study shows that methane producing status does not alter SCFA production patterns during in vitro fermentation with rhamnose, cornstarch, guar and ileostomy effluent. However, we did see a significant interaction between methane producing status and time for acetate production with lactulose. Acetate production was greater in MNP than MP early on (5 h), but the rise was delayed in MP and seen only at 24 h, probably because methanogens are slow-growing bacteria. Lactulose is fermented mainly to acetate, and in vitro work indicates that the presence of methanogens may increase acetate production (Chen and Wolin 1977, Weaver et al. 1989). It may very well be that methanogens increase acetate production only from substrates that are largely fermented to acetate in vitro. This observation has not been reported before and requires further study. It will be of interest in MP and MNP in vivo, when substrates that increase colonic acetate production are consumed.

Earlier work in a small group of subjects suggested that there are differences in fasting serum SCFA concentrations in MP and MNP; these may be due to differences in colonic production, absorption or peripheral utilization between the two groups (Wolever et al. 1993). Subsequently, it was observed that fasting serum SCFA measured in 66 MP and 63 MNP consuming regular diets were not different (Fernandes et al. 1998). Because differences in colonic production of SCFA may be difficult to observe in peripheral blood from fasting subjects, this study looked at in vitro production as an alternative way of examining differences between the two groups. Our results with the substrates other than lactulose agree with other earlier and recent studies in the literature. Wolin and Miller (1983) suggested that in humans, the amount of methane produced is rarely sufficient to influence significantly the species that produce the major fermentation products. They found no significant differences in the ratios of acetate:propionate:butyrate (56:21:19 vs. 55:22:23) in feces of producers and nonproducers and concluded that this may be due to a considerable amount of acetate being produced by homoacetate fermentation in nonproducers. In methane producers, this homoacetate fermentation is displaced by an acetate-producing methanogenic fermentation. An alternative pathway of CO_2 reduction to acetate has been shown to exist in methane nonproducers, which may compensate for the increased acetate produced in methane producers (Lajoie et al. 1988). It is becoming increasingly evident that acetate produced by homoacetate fermentation may be a major source of acetate and an important pathway for hydrogen disposal in the colon of subjects harboring low numbers of methanogens (Bernalier et al. 1996, Lajoie et al. 1988, Miller and Wolin 1996). The highest populations of acetogens were observed in the feces of methane nonproducers (Doré et al. 1995a). It has also been suggested that there might be a competitive interrelation in the human colon between methanogenic and acetogenic bacteria (Doré et al. 1995b). Studies have suggested that acetogenesis may contribute 25–33% of total acetate produced (Leclerc et al. 1997, Miller and Wolin 1996).

We also studied the effect of the different substrates (lactulose, rhamnose, cornstarch, gum guar and ileostomy effluent) on SCFA production profiles in the two groups combined. The substrates used in our in vitro system either mimicked normal dietary constituents available for fermentation in the colon (cornstarch and ileostomy effluent) or were mono- (rhamnose) or disaccharides (lactulose or 4-*O*-β-D-galactopyranosyl D-fructofuranose), and a dietary fiber supplement (gum guar). The type of substrate affected SCFA production in vitro because the monosaccharide composition of fiber largely influences SCFA production patterns (Barry et al. 1989, Mortensen et al. 1988). At 24 h, lactulose fermentation produced signif-

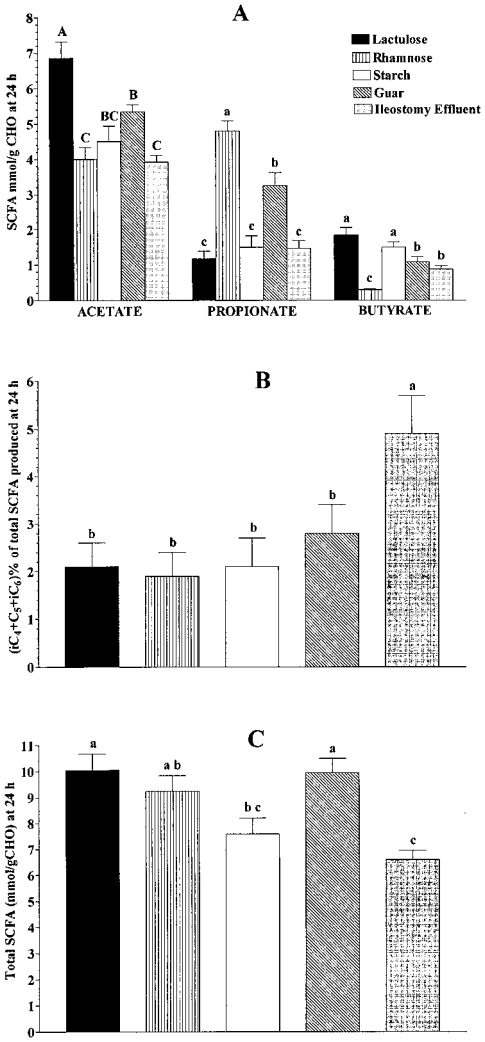

FIGURE 2 Branched-chain and total short chain fatty acids produced from lactulose, rhamnose, cornstarch, gum guar and ileostomy effluent in human feces in vitro at 24 h. *Panel A*: Acetate, propionate and butyrate produced expressed as mmol/g carbohydrate (mmol/g CHO). *Panel B*: Isobutyrate, valerate and isocaproate produced as a percentage of total SCFA in the whole group expressed as mmol/gm carbohydrate (mmol/g CHO). For *Panels A* and *B*, values are means ± SEM, $n = 11$. Means not sharing a common letter superscript are significantly different, upper case letters, $P < 0.01$; lower case letters, $P < 0.001$. *Panel C*: Total short-chain fatty acids (acetate, propionate, butyrate, isobutyrate, valerate and isocaproate) produced in the two groups combined. Values are means ± SEM, $n = 11$. Means not sharing a common letter superscript are significantly different ($P < 0.05$).

icantly more acetate than the other substrates we studied. In addition, the total production of butyrate by lactulose fermentation, as well as butyrate produced as a percentage of total SCFA at 24 h from lactulose (18.2%), was not significantly different from cornstarch (20.9%). Fermentation of cornstarch is widely known to yield more butyrate (Macfarlane and Englyst 1986, Weaver et al. 1989 and 1992), but, only one other study has reported significant butyric acid production from lactulose (Sahota et al. 1982). Butyrate is important as a source of energy to colonocytes; it regulates differentiation of cultured cells and inhibits tumor growth in vitro and possibly inflammation (Mortensen and Clausen 1996).

Another interesting finding, observed in an earlier study in humans, was that in vitro fermentation of rhamnose produced significantly more propionate (Mortensen et al. 1988). It also produced a significantly higher PR:AC ratio compared with the other substrates we studied. This observation has significance for in vivo study in view of the hypocholesterolemic effects of propionate. Studies in isolated rat hepatocytes have shown that propionate at various concentrations inhibits cholesterol synthesis (Beynen et al. 1982, Nishina and Freedland 1990, Wright et al. 1990). Rectal infusion studies show that colonic acetate is incorporated into serum cholesterol and triglycerides, acutely raises the levels of serum lipids and these effects are blocked by propionate (Wolever et al. 1991 and 1995). In animal studies, decreased serum cholesterol concentrations were observed in rats fed diets supplemented with propionate (Illman et al. 1988), or cholesterol and propionate (Chen et al. 1984). In pigs, orally administered propionate appeared to depress cholesterol synthesis only when tallow was included in the diet (Boila et al. 1981) and also lowered serum cholesterol (Thacker et al. 1981). In obese hyperinsulinemic rats, propionate induced a significant lowering of the liver cholesterol pool when propionate was fed orally (1 g/d) or infused rectally (0.15 g/d) for 19 d together with diets high in cholesterol and fat compared with a control group (Berggren et al. 1996). Feeding propionate in the form of calcium propionate (75mmol/d for 15 d) to humans with a serum cholesterol > 5.5 mmol/L reduced serum cholesterol by 5% (Stephen et al. 1994), although no effect of propionate feeding was seen in other studies (Todesco et al. 1991, Venter et al. 1990). In the context of these studies, feeding rhamnose, which is fermented largely to propionate in vitro, to humans may provide answers to a number of basic questions.

Ileostomy effluent, which contains more protein (21.8%) compared with the other substrates, produced the greatest amount of isobutyric, valeric and isocaproic acid as a percentage of total SCFA at 24 h. In humans under normal conditions, colonic fermentation leads to the production of small amounts of isobutyrate, valerate and isocaproate, and it has been suggested that they originate from protein and polypeptide breakdown (Mortensen et al. 1988, Rasmussen et al. 1988).

In conclusion, our study shows that in vitro methane producing status may affect colonic SCFA production profiles only from substrates that are largely fermented to acetate but not other substrates. It does not rule out the possibility, however, that there might be altered SCFA absorption in methane producers compared with nonproducers. This study has shown that depending on the type of substrate fermented in vitro, significantly different SCFA profiles can be produced. This finding has large implications in the planning of future studies to examine the in vivo effect of feeding these substrates to humans, altering colonic SCFA patterns and studying the potential effects on serum concentrations of SCFA and lipids.

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