Comparison of in vitro productivities of short-chain fatty acids and gases from aldoses and the corresponding alcohols by pig cecal bacteria

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We studied patterns of gas-release and short-chain fatty acids (SCFA) production from aldoses (D-glucose and D-xylose) and their alcohols (D-sorbitol and D-xylitol) by a batch culture technique using pig cecal bacteria. The hydrogenation of aldoses elongated the lag time by 3–10 hr and retarded the rate of fermentation. The productions of SCFA from aldoses (86.5 and 114.9 μmoles, respectively) were greater than those from their alcohols (40.1 and 2.5 μmoles, respectively) of the same weight (10 mg) during 8 hr of incubation and the same variance was observed during 24 hr of incubation. Productions of SCFA from pentoses were greater than those from hexoses during 24 hr of incubation ($P < 0.05$). Hydrogenation of aldoses influenced molar proportions of produced SCFA (acetic:propionic:n-butyric acids). At 24 hr of incubation the molar proportions from D-glucose and D-sorbitol were 38:55:8 and 25:71:4, respectively. These results suggest that hydrogenation of aldoses slows down the fermentation rate (production of SCFA) and that sugar alcohols may escape the microbial breakdown during the colonic transit.

Keywords: pig; large intestine; fermentation; sugar alcohols; gas-release; short-chain fatty acids

Introduction

Use of sugar alcohols such as sorbitol, mannitol, or xylitol for food and drugs is increasing.[1] Generally, only a small fraction of oligosaccharide alcohol is absorbed from the small intestine.[1,2] Thus, most of ingested sugar alcohols reach the large intestine where they are fermented to produce short-chain fatty acids (SCFA) and gases such as carbon dioxide, hydrogen, and/or methane.[3] However, actual microbial digestibility of such carbohydrates depends on the lag time and rate of fermentation, because the transit time in the active fermentation site in the human large intestine is relatively short (ca. 10 hr).[4] Each substrate has its own characteristic time-course of fermentation[5] and the inherent pattern of SCFA production.*†‡[6,7] However, the relationship between the chemical structure and fermentation characteristics of sugars is not yet fully known.

Besides energetic contributions to the host[8,9] and to colonocytes,[10] physiological influences of SCFA on epithelial cell proliferation[11] and intestinal motility[12] have become evident. Such influences vary among acids and are rate dependent. Accordingly, it is important to know the mode of production of SCFA from substrates for hind gut fermentation.

The purpose of the present study was to characterise the fermentation of aldoses (D-glucose and D-xylose) and their alcohols (D-sorbitol and D-xylitol) by monitoring the time-courses of their fermentations.

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†Key, F.B. and Mathers, J.C. (1987). Response of rat caecal metabolism to varying proportions of white and wholemeal bread. *Proc. Nutr. Soc.* **46**, 11A (abstr.)

‡Key, F.B. and Mathers, J.C. (1988). Response of rat caecal metabolism to white and wholemeal breads given at two fat levels. *Proc. Nutr. Soc.* **47**, 101A (abstr.)

Methods and materials

Culture apparatus

The mini-scale batch culture technique of Kikuchi and Sakata§ was adopted with minor modifications (*Figure 1A*). A 4 mL glass test tube (Iwaki, Tokyo, Japan) with a joint stopper (*Figure 1B*) was used as the culture vessel. An 18G-1½ injection needle (1.25 × 38 mm) (Terumo, Tokyo, Japan) inserted into two layers of Tygon tubing (inner diameter of 2.38 mm, outer diameter of 5.56 mm) (Norton, Akron, OH, USA) was connected to a silicone stopper (*Figure 1B*). A small amount of marker solution (75% $MgCl_2$ [wt/vol, 7.88 mol/L] containing 0.05% methyl orange [wt/vol, 0.0015 mol/L]; pH was adjusted to below pH 3 with HCl) (Wako Pure Chemical Industries, Tokyo, Japan) was placed in a 2 mL glass pipet connected to the test tube with Tygon tubing and the needle. The marker solution was acidified to prevent the dissolution of released carbon dioxide. The fermentation vessel was filled with CO_2 before the incubation.

Preparation of inoculum

Fresh cecal contents (approximately 600 g each) from seven 6–7-month old pigs were sampled within 30 minutes after slaughter. The cecal contents of each pig were diluted with an equal volume of bicarbonate buffer ($NaHCO_3$, 9.240 g; $Na_2HPO_4 \cdot 12H_2O$, 7.125 g; NaCl, 0.470 g; KCl, 0.450 g; $CaCl_2 \cdot 2H_2O$, 0.073 g; $MgCl_2 \cdot 6H_2O$, 0.087 g/L) (Wako) within 3 hr after the sampling.[13] The pH of the buffer was adjusted to 7.0 by bubbling with CO_2 gas before use. The diluted contents were filtered through four layers of gauze. Each pellet of washed bacteria was prepared from the filtrate by centrifuging.[14] Then each pellet of bacteria was stored at 4° C in a gas-tight vessel filled with CO_2 gas until use. The stored pellet was incubated without buffer at 25° C overnight before use. The pre-incubation was done to exhaust substrates possibly remaining in the pellet.

Substrates and buffer

We used two aldoses and their alcohols as model substrates. D-glucose (Wako) and D-xylose (Towa Chemical Industry, Tokyo, Japan) were used as representatives of hexose and pentose, respectively. D-sorbitol and D-xylitol (Towa) were also used as representatives of their reduced forms.

Preparation of the culture

One gram of bacterial pellet from each pig was mixed with 5 mL of bicarbonate buffer[13] saturated with CO_2 gas. Ten mg of a substrate was added to 0.5 mL of bacterial suspension. Then, 0.5 mL of the buffer without bacteria was added to the suspension and mixed by bubbling with CO_2 gas in the test tube. The test tube was connected to the pipet as stated above (*Figure 1B*). Every three vessels for a given substrate were incubated in a water bath at 37° C. One of them was sampled at 8 hr. The other two were incubated for 24 hr.

Measurement of gas release

We recorded the movement of the marker solution in the pipet using a video recorder equipped with an internal clock (VC-N86, NEC, Tokyo, Japan) and a color video camera (CCD-F330, Sony, Tokyo, Japan). The gas release was monitored by watching the movement of the marker solution in the pipet on the playback of the video tapes. Thus measured volumes of released gas were corrected for blank (a culture without the substrate).

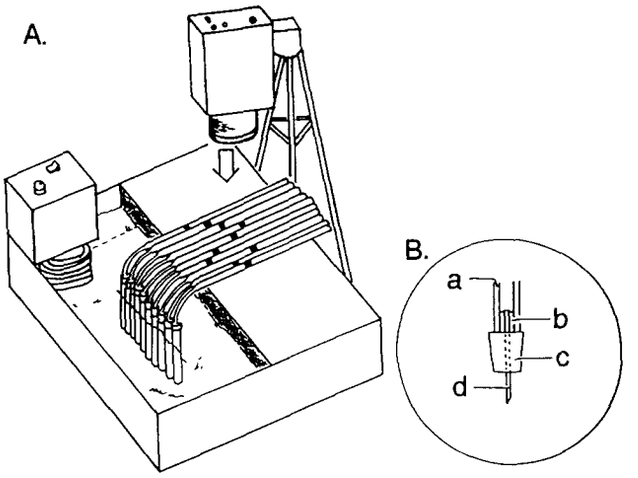

Figure 1 A: Illustration of the modified mini-scale batch culture system. B: Construction of a joint stopper. a, outer Tygon tubing (I.D.2.38 and O.D.5.56 mm); b, inner Tygon tubing (I.D.0.79 and O.D.2.38 mm); c, silicone stopper; d, 18G-1½ injection needle (1.25 × 38 mm).

Calculation of gas release rate

Because the reading tended to oscillate, rates of gas release were smoothed by calculating moving averages using the following equation: Gas release rate at the time of t (mL/h) = {rate at (t − 1) + 2 × rate at t + rate at (t + 1)}/4.

Analysis of short-chain fatty acids

At the end of incubation we measured the pH of the culture and immediately centrifuged the culture at 35,000*g* at 4° C for 25 min. The supernatant was filtered through a Molle-Cut filter (limiting m. w. 10,000, UFP1 TGC BK, Japan Millipore Industry, Tokyo, Japan). The prepared samples were stored at −20° C until analysis. SCFA were analyzed by high performance liquid chromatography (LC-6A, Shimadzu, Kyoto, Japan) with a differential refractive index detector (RID-2A, Shimadzu) equipped with an HPX-87H column (300 × 7.8 mm, Bio-Rad, Richmond, CA, USA). The column temperature was maintained at 45° C. The flow rate of carrier solution (0.005 mol/L H_2SO_4) was 0.6 mL/min. Concentrations of SCFA (acetic, propionic, and n-butyric acids) were calculated by an external standard method on a Chromato pack computer (20 C-R6A, Shimadzu). The values were corrected for blank values.

Preparation of the external standard solution of SCFA

Acetic, propionic, and n-butyric acids (Nacalai Tesque, Inc., Kyoto, Japan) were mixed and adjusted to 10 μmol/L. The standard mixed solution was stored at −20° C until use.

§Kikuchi, H. and Sakata, T. (1988). Bioassay system for dietary fibers on mini-scale batch cultures of using large intestinal bacteria. *Nippon Eiyo Shokuryo Gakkaishi* **42**, p14 (abstr.)

Statistical analysis

Differences between means were tested by Duncan's multiple-range test after preliminary analysis of variance (ANOVA). The difference was considered significant when probability was smaller than 0.05.

Results

Effects of chemical structure on gas release

Figure 2 shows that the chemical structure of aldose and their alcohols influenced the time-course of gas release. D-glucose and D-xylose showed similar time-courses of gas release, while the time-courses of gas release varied between D-sorbitol and D-xylitol. Gas release from aldoses started earlier than those from sugar alcohols. Preliminary ANOVA showed a significant three-way interaction (hydrogenation (A) × number of carbons (B) × time) ($P < 0.05$). Thus, results were analyzed by two-way ANOVA (A × B) at each time point. There were significant A × B interactions from 9–16 hr of incubation, when gas release from D-sorbitol was higher than from D-xylitol ($P < 0.05$ by Duncan's test). The effect of number of carbons on gas release was not significantly different except at 13 hr of incubation. Hydrogenation of aldose depressed gas release during 2–6 hr of incubation ($P < 0.005$ or 0.01). The start of gas releases from hydrogenated aldoses (D-sorbitol and D-xylitol) was slower than that from aldoses (D-glucose and D-xylose) ($P < 0.05$ by Duncan's test).

Effects of chemical structure on the peak time of gas release rate

Table 1 shows that the chemical structure of aldose and their alcohols influenced the peak time of gas release rate. There was significant A × B interaction for peak times of gas release rate ($P < 0.005$). Peak times of gas release rates for aldoses were faster than those for their alcohols ($P < 0.005$). Aldoses reached the peak of gas release rate at about 5 hr of incubation, whereas, their alcohols reached later. Peak times of gas release rates from pentoses were later than those from hexoses ($P < 0.005$). Effect of number of carbons on peak time of gas release rate was observed between only the alcohol of hexose and pentose ($P < 0.05$ by Duncan's test).

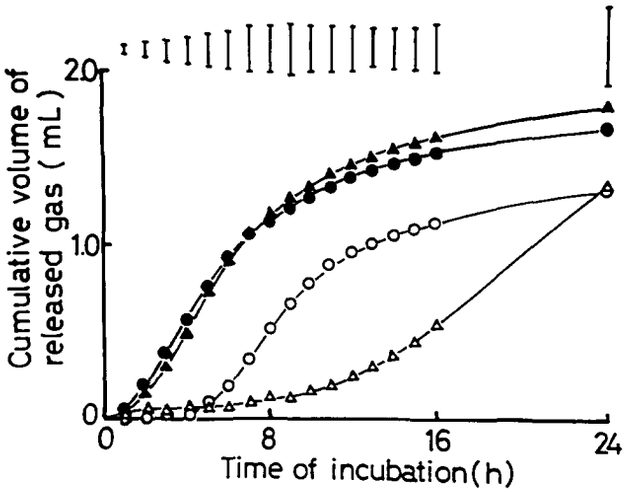

Figure 2 Effects of hydrogenation [A: (D-glucose and D-xylose) versus (D-sorbitol and D-xylitol)] and number of carbons [B: pentose-type (D-xylose and D-xylitol) versus hexose-type (D-glucose and D-sorbitol)] on time-courses of gas-release from 10 mg of D-glucose (-●-), D-sorbitol (-○-), D-xylose (-▲-) and D-xylitol (-△-) in the batch cultures using pig cecal bacteria. The vertical bars show the pooled SEM (standard error of the mean) at each time point (error degree of freedom = 13).

Influences of hydrogenation and number of carbon on SCFA production

At 8 hr and 24 hr of incubation, the production of SCFA from blank were only 26.5 ± 1.9 and 27.3 ± 8.0 μmoles ($n = 4$), respectively. There were significant A × B × SCFA (three levels: acetic, propionic, and n-butyric acids) interactions at 8 hr and 24 hr of incubation ($P < 0.05$ and 0.01, respectively) (*Table 2*). Thus, results for those at 8 hr and 24 hr were analyzed separately.

At 8 hr of incubation, there were significant A × B interactions on acetic, propionic, and n-butyric acids and total SCFA production ($P < 0.05$). SCFA pro-

Table 1 Effect of hydrogenation [A: (D-glucose and D-xylose) versus (D-sorbitol and D-xylitol)] and number of carbons[B:pentose-type (D-xylose and D-xylitol) versus hexose-type (D-glucose and D-sorbitol)] on peak time of gas-release rate in the batch culture using pig cecal bacteria (Error degree of freedom = 13).

	Hexose type		Pentose type			Factor		
Substrate	D-glucose ($n = 4$)	D-sorbitol ($n = 4$)	(h) D-xylose ($n = 5$)	D-xylitol ($n = 4$)	Pooled SEM*	A	B	A × B
Peak Time	5.0^{c}	8.3^{b}	4.8^{c}	15.0^{a}	±1.3	†	†	†

*SEM, Standard error of the mean.
†, Significantly different by two-way analysis of variance ($P < 0.005$).
n, number of pigs.
^{abc}, means not sharing a common superscript differ significantly by Duncan's multiple range test ($P < 0.05$).

Table 2 Effect of hydrogenation [A: (D-glucose and D-xylose) versus (D-sorbitol and D-xylitol)], and ring structure [B: pentose-type (D-xylose and D-xylitol) versus hexose-type (D-glucose and D-sorbitol)] on pH and productions of SCFA from 10 mg of these sugars in the batch cultures using pig cecal bacteria at 8 and 24 hr of incubation (Error degree of freedom = 14).

Substrate	Hexose Type		Pentose Type		Pooled SEM†	Results of ANOVA Factor		
	D-glucose (n = 7)	D-sorbitol (n = 4)	D-xylose (n = 3)	D-xylitol (n = 4)		A	B	A × B
	after 8 hr of incubation							
pH	6.3[c]	6.7[b]	6.1[c]	7.3[a]	± 0.2	NS	NS	NS
				(μmoles)				
Acetate	36.8[b]	7.2[c]	55.1[a]	0.9[c]	± 9.2	***	NS	**
Propionate	43.2[a]	32.4[a]	51.8[a]	1.1[b]	±13.4	***	NS	*
n-Butyrate	6.5[a]	0.5[b]	8.2[a]	0.5[b]	± 3.5	***	*	*
Total	86.5[a]	40.1[b]	114.9[a]	2.5[c]	±21.4	***	NS	*
	after 24 hr of incubation							
pH	6.4	6.7	6.3	6.4	± 0.2	NS	NS	NS
				(μmoles)				
Acetate	34.2[b]	18.4[c]	45.0[ab]	53.7[a]	±10.8	NS	***	*
Propionate	48.8	52.6	61.8	43.9	±12.7	NS	NS	NS
n-Butyrate	6.7[a]	3.2[a]	6.6[a]	0.7[b]	± 3.0	***	NS	NS
Total	89.7[b]	74.1[c]	113.4[a]	98.2[b]	± 9.6	*	***	*

†SEM, Standard error of the mean.
***, **, *: Significantly different by two-way analysis of variance ($P < 0.005$, 0.01, and 0.05, respectively).
NS, not significant ($P > 0.05$).
n, number of pigs.
[abc], Means in a row not sharing a common superscript differ significantly by Duncan's multiple range test ($P < 0.05$).

ductions were remarkably depressed by hydrogenation of aldose ($P < 0.005$). SCFA production from D-glucose was higher than that from D-xylose. The variance between hexose-type and pentose-type was not apparent in sugar alcohols.

At 24 hr of incubation, there were significant A × B interactions on only total SCFA and acetic acid productions. Total SCFA production from aldoses still exceeded that from the alcohols at 24 hr of incubation. Number of carbons also influenced total SCFA production. Acetic acid productions from pentoses were more than those from hexoses. Hydrogenation of pentose reduced n-butyric acid production ($P < 0.05$), whereas, hydrogenation of hexose did not change n-butyric acid production.

Proportion of SCFA production

Each SCFA produced from D-glucose and D-sorbitol was in the order of propionic > acetic > n-butyric acids at 8 hr and 24 hr of incubation (*Table 2*). The proportion of propionic acid from D-sorbitol was high (above 70%) at 8 hr and 24 hr of incubation. The proportion of SCFA production from D-xylose were in the order of acetic > propionic > n-butyric acids at 8 hr of incubation. However, production of propionic acid exceeded that of acetic acid at 24 hr. The proportion of SCFA production from D-xylitol was in the order of acetic > propionic > n-butyric acids at 24 hr of incubation. Pentoses produced more acetic acids than hexoses on a molar basis ($P < 0.005$) at 24 hr of incubation.

Discussion

The present study indicated that sugar alcohols were fermented more slowly and after longer lag time than their corresponding aldoses with the longer lag time. Xylitol was extremely slowly fermented with giving gas and SCFA productions, even at 24 hr of incubation.

The correlation between the volume of SCFA production (X) and released gas (Y) was significant ($Y = 0.01X + 0.17$, $r^2 = 0.6856$, $P < 0.001$, $df = 33$) in the present study. This agrees with the results of Kikuchi and Sakata§ ($Y = 0.025X - 0.36$, $r^2 = 0.96$, $P < 0.001$). Both results may suggest that the time-course of gas release can reflect that of SCFA production. Further, the above agreement implies the methodological stability of the batch culture. Moreover, this method can treat 20 samples and record the change in gas release without workers watching.

In this study, we used pig cecal bacteria as inoculum instead of rat cecal contents as in Kikuchi and Sakata§. The pig is an omnivore having body weight comparable to that of humans. Intestinal microflora of the pig resemble those of humans.[15,16] Thus, the present system using pig cecal bacteria may be validated as a simulation of human large intestine.

The bacterial pellet used in the present study contained about 2.5×10^{11} anaerobic colony forming unit/

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g wet weight of pellet¶. Although the initial redox potential of bacterial suspension without substrate was -103.3 ± 10.2 mV (mean ± SEM, $n = 3$), at 5 min of preparation, the potential immediately decreased below -200 mV (-259 mV, $n = 2$). The potential was -323.0 ± 17.2 mV ($n = 3$) at 15 min of preparation¶. These redox potentials enable us to grow bacteroides.

Batch cultures using diluted whole feces usually produce considerable amounts of SCFA even in blank cultures‡. In contrast, the production of SCFA from blank culture was small (27.3 ± 8.0 μmoles) in the present study. Thus, the use of washed bacteria in the present study should have markedly reduced the contamination substrates accompanying the inoculum, and thereby allowed more accurate evaluation of substrates of interest.

Both exhaustion of substrates and accumulation of products may limit fermentation in a batch culture. However, the final pH of the culture (above 6.3) (*Table 2*) in this study indicated that the accumulation of end products was not serious. The exhaustion of substrates most likely accounts for the flattening of curves at 24 hr (*Figure 2*).

Total SCFA productions at 24 hr after the incubation (*Table 2*) were almost equal to the results reported by previous authors†.[5,6,8] This may also support the validity of the present method.

Metabolic adaptation of bacteria would be responsible for the longer lag time before the fermentation of sugar alcohols. Aldoses D-glucose and D-xylose are normally present in the cecum of pigs fed a normal diet. On the other hand, sugar alcohols such as D-sorbitol and D-xylitol are rarely included in normal diets for pigs. Accordingly, it is unlikely that indigenous bacterial populations of pigs had adapted to such sugar alcohols. D-xylitol was fermented slower than D-sorbitol in the present study (*Figure 2*). Krishnan et al.[17] and Wallace[18] reported that xylitol was more resistant than sorbitol to non-adapted bacteria. Their results and our results suggest that number of carbons can effect fermentability.

SCFA absorbed from the large bowel enter the systemic energy pool of the host animal. The rate of SCFA production is important when one considers the energetic contribution of non-digestible carbohydrates that undergo hindgut fermentation. Because the transit time in the site of active fermentation is only 10 hr in humans,[4] non-digestible carbohydrates can escape from bacterial digestion if transit time is sufficiently slow.

The ring structure had only minor effects on SCFA production (*Table 2*). The hydrogenation of aldose had stronger effects on the fermentation than the ring structure. However, such generalization requires more experiments involving a broader spectrum of substrates. In this study, molar proportions of acetic and propionic acids depended on the number of carbons in the aldose.

¶Kiriyama, H., Hariu, Y., and Sakata, T. Unpublished data.

‡Key, F.B. and Mathers, J.C. (1988). Response of rat caecal metabolism to white and wholemeal breads given at two fat levels. *Proc. Nutr. Soc.* **47,** 101A (abstr.)

†Key, F.B. and Mathers, J.C. (1987). Response of rat caecal metabolism to varying proportions of white and wholemeal bread. *Proc. Nutr. Soc.* **46,** 11A (abstr.)

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