Stimulation of Butyrate Production by Gluconic Acid in Batch Culture of Pig Cecal Digesta and Identification of Butyrate-Producing Bacteria

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ABSTRACT Gluconic acid reaches the large intestine to stimulate lactic acid bacteria. However, the fermentation pattern of gluconic acid has yet to be elucidated. Accordingly, we examined the fermentation properties induced by gluconic acid in the pig cecal digesta in vitro. We also tested sorbitol and glucose, substrates for which the fermentation rate and patterns are known. The gluconic acid–utilizing bacteria were further isolated from pig cecal digesta and identified to examine the effect of gluconic acid on hind gut fermentation. Gluconic acid was fermented more slowly than were the other two substrates. Gluconic acid stimulated butyrate production; the butyrate molar percentage reached 26%, which is considered a high butyrate production. The majority of gluconic acid fermenters were identified as lactic acid bacteria, such as Lactobacillus reuteri and L. mucosae, and acid-utilizing bacteria, such as Megasphaera elsdenii and Mitsuokella multiacida. The gluconic acid fermented by lactic acid bacteria, and the lactate and acetate that were produced were used to form butyrate by acid-utilizing bacteria, such as M. elsdenii. Gluconic acid may be useful as a prebiotic to stimulate butyrate production in the large intestine. J. Nutr. 132: 2229–2234, 2002.

KEY WORDS: • gluconic acid • butyrate enhancer • Megasphaera elsdenii • lactic acid bacteria • pig cecal digesta

Short-chain fatty acids (SCFA2), such as acetate, propionate and n-butyrate, are the main end products of fermentation in the large intestine (1). SCFA are rapidly absorbed from the mucosa of the large intestine (2) to provide energy to epithelial cells (3). Among SCFA, butyrate is considered to be the most effective for promoting epithelial growth (4,5) and sodium and water absorption (6). Moreover, the inhibitory effect of butyrate on tumor development has been frequently discussed (7–9). Stimulation of butyrate production in the hindgut by means of dietary components is thus thought to be desirable.

Many saccharides are indigestible materials that reach the large intestine (10–17). However, in only a few cases have saccharides been shown to stimulate butyrate production in the large intestine (11–13,17). We are, therefore, seeking novel stimulators of butyrate production. It has been shown that gluconic acid, which may be fermented in the large intestine, can reach the large intestine of rats (18). However, the fermentation pattern induced by gluconic acid has not been elucidated.

Accordingly, the aim of the present study was to investigate the fermentation properties induced by gluconic acid in a batch culture of pig cecal digesta. Glucose and sorbitol were also tested because these sugars are structurally related to gluconic acid and their fermentations have been studied previously in in vitro human fecal and pig cecal models (14,17).

According to these reports, sorbitol can reach the large intestine and may stimulate butyrate or propionate production. The fermentation rate, which may be a factor that affects the fermentation pattern of these sugars, was also reported (14). We thus compared the effect of gluconic acid with that of sorbitol (slowly fermented sugar) and glucose (rapidly fermented sugar) on fermentation in the large intestine.

Gluconic acid–utilizing bacteria were isolated from pig cecal digesta and identified to explain how gluconic acid was used and metabolized. Their roles in gluconic acid fermentation and, particularly, in butyrate production were investigated. Pigs are often considered to be one of the better human nutritional and physiological models in terms of the large intestine (19–23). Therefore, the possible effects of gluconic acid on fermentation in the large intestine of humans were also investigated in the present study.

MATERIALS AND METHODS

Animals and diet

Three adult cross-bred (Landrace × Large white × Duroc) sows weighing 185, 180 and 163 kg, fitted with permanent cecal cannulas, were used as donors of the cecal digesta. The pigs were fed 500 g meat-bone meal, 500 g alfalfa (Medicago sativa) meal and 1640 g cracked maze supplemented with 8 g NaCl, 14 g CaCO3 and 8 g vitamin–mineral premix (Kokin Kagaku, Osaka, Japan) daily, as
described (24). Pigs consumed the food 3 completely and drank water ad libitum.

**Chemicals**

All chemicals were obtained from Wako Pure Chemicals (Osaka, Japan) and Nacalai tesque (Kyoto, Japan) unless otherwise stated.

**Experiment 1**

Sodium gluconate (Fujisawa Pharmaceutical, Tokyo, Japan), glucose, and soytanol were used as substrates in this experiment. The diluting procedure was the same as that used by Ushida and Sakata (26). Briefly, cecal digesta were sampled from three sows by suction through the cannula, just after morning feeding, and diluted with four volumes of an anaerobic sodium phosphate buffer (50 mmol/L, pH 6.5). Diluted digesta were squeezed through four layers of surgical gauze. A portion (25 mL) of strained digesta was inoculated into a 120-mL serum bottle that contained substrate. The initial concentration of the substrate was adjusted to 40 mmol/L. The bottles were incubated at 37°C without gas phase for 24 h. Fermentation was stopped by the addition of 1 mL of 6 mol/L HCl. After centrifugation at 12,000 × g for 15 min, the organic acid concentrations of the supernatant were analyzed by ion-exclusion HPLC (26). The apparent production of organic acids from the substrates was calculated by subtraction of the 0-time concentration from that at the end of the incubation. The blank incubations were used to evaluate the effect of endogenous substrates. The values of apparent production for the blank incubations were subtracted from those with experimental substrates.

**Experiment 2**

Cecal digesta were sampled and squeezed as in expt. 1. Squeezed digesta were diluted with nine volumes of an anaerobic dilution solution (27). A portion (1 mL) of strained digesta was inoculated into 120-mL serum bottles with 24 mL of peptone yeast extract broth containing Fildes solution (PYF broth, pH 7.0) (28). Two bottles were allotted to each pig. The bottles were filled with a gas phase at 100% CO2 and closed with butyl rubber stoppers and aluminum clips. Blank cultures (without substrate) were also performed to evaluate the organic acid production from the endogenous substrate in the sampled cecal digesta. The bottles were incubated at 39°C for 0, 4, 8, 12 and 24 h, and were laid on their sides to increase the surface of the sediment. Three bottles were allotted to each incubation period; therefore, 15 bottles were used in total for each pig, and 45 bottles in total were used in this experiment. All bottles were agitated manually at the time of sampling. At the end of the incubation, fermentation was stopped by the addition of 1 mL of 6 mol/L HCl. After centrifugation at 12,000 × g for 15 min, the organic acid concentrations of the supernatant were analyzed by ion-exclusion HPLC (26). The apparent production of organic acids from the substrates was calculated by subtraction of the 0-time concentration from that at the end of the incubation. The blank incubations were used to evaluate the effect of endogenous substrates. The values of apparent production for the blank incubations were subtracted from those with experimental substrates.

**Experiment 3**

A strain of the gluconic acid–utilizing bacteria obtained from expt. 2 was used to examine the utilization of lactate and acetate in the production of butyrate. The standard strain of *Megaspheara eldonii* JCM 1772T was also used for comparison. These two bacteria were preincubated at 37°C for 24 h in a peptone yeast extract broth (PY broth) (30) supplemented with lactate (10 mmol/L) and acetate (10 mmol/L) or with lactate (10 mmol/L) alone. All broths in this experiment were adjusted to pH 6.8. The bacteria were transferred to the same fresh media and incubated for another 24 h. After incubation was completed, the organic acids in the culture supernatant were analyzed as described above.

**Statistical analyses**

Values in expt. 1 are given as means ± SD for individual pigs (n = 3). We first conducted repeated-measure ANOVA (3 experimental groups × sampling time), to decide whether we could pool the results of different sampling times, or whether we should analyze results from different sampling times separately. Effects of both the sampling time (P < 0.05) and the two-way interaction (experimental groups × sampling time; P < 0.05) on the concentrations of all detected organic acids were significant. Therefore, we applied statistical analysis to results of each sampling time separately. Either a one-way ANOVA or the Kruskal–Wallis test, depending on the results of the Bartlett test, was used to analyze the differences among means at each time. Dunn’s post hoc comparison (parametric or nonparametric) was used for multiple comparisons when needed.

Values in expt. 2 are given as means ± SD for the cultures of each bacteria (n = 4). Either a Student’s t test or a Welch’s t test, depending on the results of the F test, was used to analyze the differences among means. Values in expt. 3 are given as means ± SD for the cultures of each bacteria (n = 3). Either a one-way ANOVA or the Kruskal–Wallis test, depending on the results of the Bartlett test, was used to analyze the differences among means. Post hoc comparison was as in expt. 1. The difference between means was considered significant at P < 0.05 in all statistical analyses. All data were analyzed by Statcel (31), which is an add-in application of Microsoft Excel (version 5.0; Microsoft, Seattle, WA).
RESULTS

Apparent organic acid production in expt. 1 (Table 1)

In all cases, irrespective of whether they increased or decreased relative to 0-h concentration, only trace amounts of succinate, formate, iso-butyrate and iso-valerate were detected. These, therefore, are acids that are not discussed in this report.

Apparent lactate production, which was detected only in the glucose culture, increased for 12 h. Apparent acetate production constantly evolved in the glucose and sorbitol cultures for 8 h, and the production rate declined for a further incubation period. Sorbitol promoted less apparent acetate production than that of glucose. It showed a sigmoidal production curve in the gluconic acid cultures; the production rate was as low as that observed in sorbitol cultures for 8 h, but it increased substantially from 12 to 24 h. Gluconic acid promoted apparent acetate production that was 20-fold higher than that of the sorbitol culture in a 24-h incubation period. Propionate was produced without a lag time in the glucose culture. The lag times for apparent propionate production were 4 and 8 h for sorbitol and gluconic acid, respectively. The final concentrations of propionate were similar for the three substrates. The apparent n-butyrate production was fairly constant in the glucose and sorbitol cultures. On the other hand, after 8 h, gluconic acid promoted a much higher apparent production of butyrate that reached 61 mmol/kg, a level that was twice those observed in the glucose and sorbitol cultures. In all three cultures, the apparent n-valerate production did not increase until 12 h had passed. Sorbitol promoted an apparent substrate production that was 3-fold higher than those of the gluconic acid cultures in the 24-h incubation.

Isolation and identification of gluconic acid–utilizing bacteria from the enrichment culture in expt. 2

Eighty-four morphologically distinct colonies were isolated from the gluconic acid–supplemented cultures. They were analyzed for gluconic acid utilization in the PYF broth. Ten bacteria grew in the gluconic acid–supplemented PYF broth. When these isolates were further tested for their ability to produce larger amounts (P < 0.05) of organic acid in gluconic acid–supplemented PYF broth than in that without gluconic acid, seven isolates were obtained (Table 2). Isolates 2, 9 and 10 did not produce significantly larger amounts of organic acid from gluconic acid. Therefore, these isolates were not subjected to further analysis. Isolate 1, whose number in the culture (log CFU/mL) was 6.8, produced lactate and acetate from gluconic acid. Isolate 3 produced formate, acetate and n-butyrate, and its number was 6.3. Isolate 4 produced lactate, formate and acetate, and its number was 9.6. Isolates 5, 6 and 7 all produced lactate and acetate, and their numbers were all 9.3. Isolate 8 produced succinate, lactate, acetate, propionate and n-butyrate from gluconic acid, and its number was 7.3.

Among the isolates, 1, 3, 4 and 8 were analyzed for their 16S rDNA partial sequence (27–1492 in E. coli position). According to cell morphology, such as the Gram-positive stained rod, and to their fermentation end products, isolates 1, 3, 4, 5, 6 and 7 appeared to be Lactobacillaceae. Therefore, isolates 1 and 4 were subjected to SSU rDNA analyses. Isolate 1 was identified as Lactobacillus reuteri (98% homology with L. reuteri DSM 20016T, GenBank Accession No. X76328); isolate 3 was Megasphaera elsdenii (98% homology with M. elsdenii ATCC 17752, GenBank Accession No. M26493); isolate 4 was Lactobacillus mucosae (96% homology with L. mucosae, GenBank Accession No. AF243148); and isolate 8 was Mitsuokella multistriata (98% homology with M. multistriata, GenBank Accession No. X81878).

Fermentation end products in Megasphaera elsdenii pure culture (Table 3)

M. elsdenii JCM 1772T and isolate 3 were used to examine their ability to use lactate and acetate for butyrate production. Lactate supplementation stimulated apparent succinate, formate, acetate, propionate and valerate production (P < 0.05) of organic acid in gluconic acid. Therefore, these isolates were not subjected to further analysis. Isolate 1, whose number in the culture (log CFU/mL) was 6.8, produced lactate and acetate from gluconic acid. Isolate 3 produced formate, acetate and n-butyrate, and its number was 6.3. Isolate 4 produced lactate, formate and acetate, and its number was 9.6. Isolates 5, 6 and 7 all produced lactate and acetate, and their numbers were all 9.3. Isolate 8 produced succinate, lactate, acetate, propionate and n-butyrate from gluconic acid, and its number was 7.3.
apparent \( n \)-butyrate production \((P < 0.05)\) in \( M. elsenii \) JCM 1772\(^T\). On the other hand, isolate 3 was less able to utilize lactate, and a relatively small increase \((P = 0.17 \text{ PYL vs. PY, } P = 0.19 \text{ PYLA vs. PY})\) in butyrate was detected.

### DISCUSSION

Acetate, butyrate and propionate apparently were produced from gluconic acid and the amount of the acetate and butyrate were the highest among the substrates tested in vitro. In the present experimental system, cecal digesta were sampled from pigs that did not receive gluconic acid as a feed additive. Therefore, the results from expt. 1 reflected the short-term effect of gluconic acid on fermentation in the large intestine. In previous studies, sorbitol elevated propionate and butyrate productions, respectively, in human fecal and in pig cecal in vitro fermentation models \((14,17)\). In this investigation, sorbitol elevated production of propionate, rather than butyrate, and this is consistent with Kiriya et al. \((14)\). The effect of sorbitol on butyrate production remains inconclusive and needs further in vivo evaluation. The effect of gluconic acid had been confirmed by an in vivo feeding trial. We observed an increase in butyrate concentration in the cecal contents \((9.6 \text{ to } 30.0 \text{ mmol/kg digesta})\) when pigs \((n = 3)\) were fed a diet supplemented with 0.5 g/100 g gluconic acid for

### TABLE 2

**Organic acid concentration of the gluconic acid-utilizing bacteria in the PYF broth with or without gluconic acid\(^1\)**

<table>
<thead>
<tr>
<th>Colony no.</th>
<th>Gluconic acid supplement(^2)</th>
<th>Succinate</th>
<th>Lactate</th>
<th>Formate</th>
<th>Acetate</th>
<th>Propionate</th>
<th>( n )-Butyrate</th>
<th>( n )-Valerate</th>
<th>Total organic acids(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>0.39 ± 0.05</td>
<td>12.66 ± 4.21*</td>
<td>0.05 ± 0.10</td>
<td>6.79 ± 2.16*</td>
<td>0.97 ± 1.14</td>
<td>1.19 ± 0.81</td>
<td>0.19 ± 0.14</td>
<td>22.58 ± 7.41*</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>0.49 ± 0.08</td>
<td>0.98 ± 0.31</td>
<td>0.20 ± 0.14</td>
<td>0.74 ± 0.19</td>
<td>1.67 ± 1.12</td>
<td>2.57 ± 0.43</td>
<td>0.58 ± 0.39</td>
<td>7.66 ± 1.75</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>3.34 ± 0.71</td>
<td>0.11 ± 0.08</td>
<td>4.42 ± 1.18*</td>
<td>9.47 ± 1.02*</td>
<td>0.49 ± 0.57</td>
<td>3.95 ± 1.19*</td>
<td>0.42 ± 0.21</td>
<td>22.39 ± 2.87*</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>4.02 ± 0.36</td>
<td>0.51 ± 0.20</td>
<td>2.71 ± 0.35</td>
<td>6.83 ± 0.72</td>
<td>1.29 ± 0.22</td>
<td>1.63 ± 0.65</td>
<td>0.19 ± 0.14</td>
<td>17.43 ± 1.41</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>0.56 ± 0.16</td>
<td>28.94 ± 2.44*</td>
<td>1.22 ± 0.22*</td>
<td>16.09 ± 2.20*</td>
<td>1.32 ± 1.57</td>
<td>0.78 ± 0.93</td>
<td>0.51 ± 0.12</td>
<td>48.93 ± 4.88*</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>0.45 ± 0.03</td>
<td>1.44 ± 0.39</td>
<td>0.25 ± 0.03</td>
<td>0.99 ± 0.28</td>
<td>0.63 ± 0.60</td>
<td>0.63 ± 0.69</td>
<td>0.40 ± 0.04</td>
<td>6.52 ± 1.86</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>0.58 ± 0.11</td>
<td>29.02 ± 3.02*</td>
<td>ND(^4)</td>
<td>15.00 ± 1.80*</td>
<td>1.77 ± 1.44</td>
<td>ND</td>
<td>0.69 ± 0.29</td>
<td>47.67 ± 3.83*</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>0.44 ± 0.04</td>
<td>1.31 ± 0.36</td>
<td>0.15 ± 0.01</td>
<td>1.14 ± 0.17</td>
<td>2.61 ± 0.29</td>
<td>0.76 ± 0.30</td>
<td>0.55 ± 0.30</td>
<td>7.76 ± 0.79</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>0.72 ± 0.11</td>
<td>29.00 ± 1.95*</td>
<td>ND</td>
<td>17.21 ± 1.01*</td>
<td>2.98 ± 0.09</td>
<td>ND</td>
<td>0.57 ± 0.29</td>
<td>51.68 ± 2.79*</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>0.44 ± 0.04</td>
<td>0.90 ± 0.07</td>
<td>0.21 ± 0.05</td>
<td>0.97 ± 0.17</td>
<td>2.51 ± 0.22</td>
<td>ND</td>
<td>0.22 ± 0.04</td>
<td>6.73 ± 0.37</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>0.48 ± 0.11</td>
<td>26.12 ± 2.05*</td>
<td>ND</td>
<td>14.97 ± 0.50*</td>
<td>2.92 ± 0.23</td>
<td>ND</td>
<td>0.30 ± 0.12</td>
<td>46.89 ± 2.34*</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>0.45 ± 0.02</td>
<td>0.91 ± 0.07</td>
<td>0.16 ± 0.10</td>
<td>0.90 ± 0.04</td>
<td>0.80 ± 0.07</td>
<td>0.76 ± 0.17</td>
<td>0.06 ± 0.10</td>
<td>4.90 ± 0.49</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>0.53 ± 0.10*</td>
<td>2.78 ± 0.19*</td>
<td>0.09 ± 0.06</td>
<td>9.60 ± 0.88*</td>
<td>44.65 ± 2.84*</td>
<td>1.02 ± 0.08</td>
<td>0.34 ± 0.10</td>
<td>59.16 ± 3.11*</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>0.03 ± 0.03</td>
<td>0.48 ± 0.08</td>
<td>0.16 ± 0.03</td>
<td>2.56 ± 0.30</td>
<td>6.71 ± 0.35</td>
<td>0.26 ± 0.03</td>
<td>0.40 ± 0.04</td>
<td>10.95 ± 0.73</td>
</tr>
</tbody>
</table>

\(^1\) Values are means ± so, \( n = 4. \) * Different from blank (−) culture, \( P < 0.05 \).
\(^2\) +, Gluconic acid (46 mmol/L) was supplemented to the PYF broth.
\(^3\) See Table 1.
\(^4\) ND, not detected.
study, Lactobacilli were isolated from the gluconic acid–humans, it was demonstrated that dietary gluconic acid in-bacteria were not stimulated by dietary gluconic acid. In as a major end product of fermentation (30). However, these bacteria increased significantly; Lactobacilli and Bifidobacteria (32). In the present study, Lactobacilli were isolated from the gluconic acid–en-riched cultures as a major gluconic acid utilizer (Table 2). Obviously, Bifidobacteria and Lactobacilli do not produce butyrate (33). A similar result was published concerning the effect of resistant starch on fecal flora composition and the SCFA pattern in rats (34). In that report, butyrate production was significantly increased (7 times) by diets containing resistant starch. At the same time, the numbers of lactic acid bacteria increased significantly; Lactobacilli and Bifidobacteria increased 100 and 10 times, respectively. The relationship between increases in lactic acid bacteria and butyrate concentration seems to be indirect. It is plausible that an increased number of lactic acid bacteria induced a higher lactate production and that lactate was then efficiently converted to butyrate by acid-utilizing bacteria, such as M. elsdenii.

In the present study, M. elsdenii was indeed isolated as a unique colony from gluconic acid–enriched cultures, and their butyrate production was stimulated by lactate and acetate (Tables 2 and 3). This hypothesis was not fully substantiated in the present experiment, although it explains the following apparent contradiction about the effect of many bifidogenic prebiotics on colonic and cecal SCFA production patterns. The increase in lactic acid–producing bacteria did not induce lactic acid fermentation in the large intestine in most cases (11,35–37) and often stimulated butyrate production (11).

The fermentation rate of the substrate may be a key character-istic to determine the pattern of fermentation end products. In the present study, glucose was rapidly fermented in the batch culture of pig cecal digesta, whereas sorbitol and gluconic acid were fermented relatively slowly (Table 1). Lactate was produced at a higher rate than the lactate conversion rate during the first 5 h in the glucose culture. Rapid fermentation of glucose might cause rapid growth of Gram-positive cocci, such as Streptococci, which cause the accumulation of lactate (38). Rapid accumulation of lactate often eliminates the cca-coonconversion system because of the decline in pH (26,38). Accumulation of lactate started at pH 5.8 under conditions similar to those in this study (26). Because we added HCl to stop fermentation promptly, pH could not be measured. Accumulation of lactate at 4 h suggested a low pH at this point. Based on the lag times, sorbitol was fermented more slowly than glucose. Sorbitol was previously shown to be a slowly fermented saccharide, but gluconic acid was fermented far more slowly than was sorbitol. The production rate of each SCFA, low-passaged the clear presence of a lag time for the metabolism of gluconic acid. In preliminary experiments, the pH of cultures was as low as 6.3 when gluconic acid or sorbitol was supplemented to the cultures (Tsukahara et al., unpub-

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LITERATURE CITED


