Lactobacillus casei Strain Shirota-fermented Milk Stimulates Indigenous Lactobacilli in the Pig Intestine

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Summary The aim of this study was to determine the effect of a probiotic, i.e. fermented milk prepared with Lactobacillus casei strain Shirota, on indigenous Lactobacilli in the pig large intestine. This fermented milk was given as a probiotic to experimental pigs for 2 weeks. The fecal organic acid concentration increased with the fermented milk; acetate and propionate increased significantly (p<0.05). At the same time, lactate and butyrate tended to increase. The fecal pH was significantly reduced by the fermented milk (p<0.05). Although the number of bacteria of strain Shirota in the intestinal contents was much smaller than those of indigenous Lactobacilli, 10^4 vs 10^8 (cfu/g), the numbers of indigenous Lactobacilli and Bifidobacteria in the pig intestine appeared to increase with the fermented milk. In addition, the phenotypic diversity (phenotypic group numbers) of indigenous Lactobacilli increased from 3 to 8 with the fermented milk supplementation. Thus the fermented milk affected the indigenous Lactobacillus population and constitution.

Key Words fermented milk, probiotic, Lactobacilli, intestinal flora

The colonic flora ferments undigested food materials and endogenous substances such as mucus to produce short-chain fatty acids (SCFA) that are considered to be effective for maintenance of the host’s health (1-6). However, potentially harmful compounds such as ammonia, sulfide and indole are also produced in the same process. It is preferable for the host that these hazards to health are not produced during colonic fermentation. The control of colonic fermentation is important in this context. Two ways are usually adopted for such control; one is the administration of live bacteria, i.e. Bifidobacteria and Lactobacilli, and the other is the administration of a specific substrate for health-promoting bacteria, e.g. Bifidobacteria. The former is categorized as a probiotic and the latter as a prebiotic (7-11).

The mechanism of probiotic effect on host health has not been fully elucidated yet; some reasons such as lowering of the digesta pH due to lactate accumulation and the bactericidal effect of bacteriocin, both produced by probiotic strains, are considered to be responsible for improvement of the colonic environment (12, 13). However, these effects must not only rely on administered probiotic strains, because indigenous bacteria belonging to the same species or genera as probiotic strains may contribute to such improvement if their activity is stimulated by probiotics.

Therefore, we investigated the effect of probiotic, i.e. fermented milk prepared with Lactobacillus casei strain Shirota, on indigenous Lactobacilli in the pig large intestine.

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MATERIALS AND METHODS

Experimental design and feeding of L. casei fermented milk. Four Landrace×Large White cross bred pigs weighing 25.3±1.0 kg were used. The pigs were fed a 750 g of meal twice a day at 10:00 and 22:00. The meal consisted of cracked maize (65.3%), soybean meal (18.7%), alfalfa meal (9.3%), wheat bran (3.7%), meat bone meal (1.9%), CaCO3 (0.5%), NaCl (0.3%) and a vitamin mineral premixture (0.3%, Ton-misshu 1). These feed ingredients were purchased from Kumiai Shiroyo Co., Ltd., Kobe, Japan. Water was accessible to the pigs at all times. The pigs were divided into two groups of A and B, which were subjected to a double reversal trial consisting of three consecutive periods every three weeks (14). The pigs were given 130 mL of commercially available fermented milk containing L. casei strain Shirota (Yakult, Yakult Honsha Co., Ltd., Tokyo, Japan) with their morning meal during the test periods.

The pigs of group A were given the fermented milk in the first and third periods. Those of group B were given the fermented milk in the second period. This amount of fermented milk (130 mL) contained over 10^{10} L. casei strain Shirota.

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All the animals were housed individually in metabolic cages and handled with due regard for their welfare, as approved by the Experimental Animal Care and Use Committee, Kyoto Prefectural University, Kyoto.

Chemical analysis of the feces. After 2 wk’s acclimatization, the total feces excreted between 10:00 and 11:00 were collected. The fecal pH was immediately measured with a pH meter (Twin pH, Horiba Seisakusho Co., Ltd., Tokyo, Japan). A portion of feces...
(about 10 g) was freeze-dried to measure the moisture content. Another portion (5 g) was suspended in 10 mL of distilled water and 1.5 mL of perchloric acid was added to eliminate protein. The suspension was centrifuged at 20,000 × g at 4°C for 10 min. The supernatant was filtered through a cellulose acetate membrane filter of 0.45 μm pore size (Toyo Roshi Kaisha Ltd., Tokyo, Japan), and then analyzed as to the organic acid content by ion-exclusion HPLC as described by Ushida and Sakata (15).

Enumeration of intestinal and fecal bacteria. At the end of the last experimental period, each pig was randomly selected from both groups. After collection of their feces from 9:00 to 11:00, the pigs were subjected to extirpation of the whole large intestine under general anesthesia with Ketamine HCl (Sankyo Co., Ltd., Tokyo, Japan). The small intestine, cecum and colon were removed, and their contents were immediately collected. The contents were mixed in plastic containers in an ice-bath. The feces and intestinal contents were subjected to serial 10-fold dilutions with an anaerobic dilution solution (16) under anaerobic conditions and inoculated onto the following agar plate media principally in the same manner as described by Tanaka et al. (17). Blood Agar (COBA) medium (18) was used to enumerate Enterobacteriaceae. Colistin-Oxolinic acid Enterococci, were decreased by feeding the fermented milk. Lactobacilli-selective (LBS) medium (Becton Dickinson and Company, Cockeysville, MD, USA) and supplemented with 5% defibrinated horse blood. Lactobacillus-selective (LBS) medium (Becton Dickinson and Company, Cockeysville, MD, USA) was used to enumerate Lactobacilli. Lactitol-LBS-Vancomycin (LLV) agar (19) was used to select L. casei strain Shirota. Trypticase soy agar (TSA) medium (Becton Dickinson and Company, Cockeysville, MD, USA) supplemented with 5% defibrinated horse blood was used to enumerate the total aerobes, Bifidobacteria and Bacteroidaceae. The latter two groups were identified from the colony and cell morphotypes as well as by Gram staining. Desoxycholate-Hydrogen sulfide-Lactose (DHL) agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 5% defibrinated horse blood was used to enumerate total anaerobes, Bifidobacteria and Bacteroidaceae. The latter two groups were identified from the colony and cell morphotypes as well as by Gram staining. Desoxycholate-Hydrogen sulfide-Lactose (DHL) agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 5% defibrinated horse blood was used to enumerate total anaerobes, Bifidobacteria and Bacteroidaceae. The latter two groups were identified from the colony and cell morphotypes as well as by Gram staining. Desoxycholate-Hydrogen sulfide-Lactose (DHL) agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 5% defibrinated horse blood was used to enumerate total anaerobes, Bifidobacteria and Bacteroidaceae. The latter two groups were identified from the colony and cell morphotypes as well as by Gram staining. Desoxycholate-Hydrogen sulfide-Lactose (DHL) agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 5% defibrinated horse blood was used to enumerate total anaerobes, Bifidobacteria and Bacteroidaceae. The later two groups were identified from the colony and cell morphotypes as well as by Gram staining.

Amplification of 16S rDNA by polymerase chain reaction was directly from the transferred colonies with primers 27f and 1522r (20). PCR was performed under the following conditions: 94°C for 3 min; 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min; and finally 72°C for 5 min. The PCR products were purified with Microspin S-400 columns (Pharmacia Biotech, Uppsala, Sweden) as recommended by the manufacturer. The purified DNA was used for 16S rDNA sequence analysis by using the ABI Prim Dye Terminator Cycle Sequencing Ready Reaction Kit with primers 926f and 1392r (20) as sequencing primers. The sequences were automatically analyzed on an ABI model 373A DNA sequencer (Applied Biosystems, Foster City, CA, USA). Data analysis was performed as indicated elsewhere (21).

Statistical analyses. The results are expressed as means with standard deviations. The chemical characteristics of feces were compared between the test and control periods according to Yoshida (14) for a double reversal trial.

Chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan), unless otherwise stated.

RESULTS

Chemical characteristics of the feces (Table 1)

Fecal pH was significantly lowered by feeding the fermented milk. The acetate and propionate concentrations were significantly increased by the fermented milk (p<0.05 and p<0.01, respectively). The butyrate and lactate concentrations also became somewhat higher, although in a statistically insignificant manner. The fecal moisture content was not affected by feeding the fermented milk.

Population sizes of intestinal bacteria in the pig intestine and feces (Table 2)

The numbers of total aerobes, Enterobacteriaceae and Enterococci, were decreased by feeding the fermented milk.

Table 1. Effects of Lactobacillus casei strain Shirota-fermented milk administration onecal pH, fecal organic acids and fecal moisture in pigs.

<table>
<thead>
<tr>
<th>L. casei strain Shirota-fermented milk administration</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fecal pH</td>
<td>6.7±0.5</td>
<td>6.0±0.2*</td>
</tr>
<tr>
<td>Lactate (mm)</td>
<td>0.3±0.3</td>
<td>0.9±0.5</td>
</tr>
<tr>
<td>Acetate (mm)</td>
<td>49.7±4.7</td>
<td>61.3±7.4*</td>
</tr>
<tr>
<td>Propionate (mm)</td>
<td>23.7±4.1</td>
<td>32.8±6.1**</td>
</tr>
<tr>
<td>Butyrate (mm)</td>
<td>11.6±3.0</td>
<td>15.5±3.0</td>
</tr>
<tr>
<td>Fecal moisture (%)</td>
<td>67.3±3.7</td>
<td>63.0±3.2</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01.

The experiment was carried out as a double reversal trial. The results are mean values for 4 pigs±SD.
Table 2. The viable cell numbers of *L. casei* strain Shirota and intestinal bacteria in the intestines of pigs administered and not administered *L. casei* strain Shirota-fermented milk (log CFU/g digesta).

<table>
<thead>
<tr>
<th>Administration</th>
<th>Intestinal bacteria</th>
<th>AE</th>
<th>EB</th>
<th>EC</th>
<th>AN</th>
<th>BI</th>
<th>BA</th>
<th>LB</th>
<th>LC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small intestine</td>
<td>4.23</td>
<td>3.23</td>
<td>3.39</td>
<td>9.26</td>
<td>8.84</td>
<td>8.62</td>
<td>8.70</td>
<td>2.90</td>
<td></td>
</tr>
<tr>
<td>Cecum</td>
<td>4.61</td>
<td>4.55</td>
<td>3.37</td>
<td>9.24</td>
<td>8.83</td>
<td>8.50</td>
<td>8.74</td>
<td>4.22</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>5.40</td>
<td>4.47</td>
<td>4.33</td>
<td>10.21</td>
<td>9.89</td>
<td>9.61</td>
<td>9.32</td>
<td>4.58</td>
<td></td>
</tr>
<tr>
<td>Non-administration</td>
<td>Small intestine</td>
<td>4.83</td>
<td>4.44</td>
<td>3.54</td>
<td>7.87</td>
<td>6.71</td>
<td>6.71</td>
<td>&lt;2.30</td>
<td></td>
</tr>
<tr>
<td>Cecum</td>
<td>5.98</td>
<td>5.97</td>
<td>3.91</td>
<td>9.06</td>
<td>7.37</td>
<td>8.56</td>
<td>8.29</td>
<td>&lt;2.30</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>7.30</td>
<td>7.07</td>
<td>5.20</td>
<td>10.02</td>
<td>7.94</td>
<td>9.66</td>
<td>8.67</td>
<td>&lt;2.30</td>
<td></td>
</tr>
<tr>
<td>Feces</td>
<td>6.78</td>
<td>6.76</td>
<td>4.78</td>
<td>9.13</td>
<td>7.24</td>
<td>8.38</td>
<td>7.93</td>
<td>&lt;2.30</td>
<td></td>
</tr>
</tbody>
</table>


Table 3. Effect of *L. casei* strain Shirota-fermented milk administration on the composition of Lactobacilli isolated from pig feces (%).

<table>
<thead>
<tr>
<th>Group</th>
<th>Identification</th>
<th>16S rDNA sequence</th>
<th>Non-administration</th>
<th>Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>L. brevis</em> 1</td>
<td><em>L. reuteri</em></td>
<td>2.9</td>
<td>6.60</td>
</tr>
<tr>
<td>2</td>
<td><em>L. brevis</em> 2</td>
<td><em>L. reuteri</em></td>
<td>68.5</td>
<td>7.76</td>
</tr>
<tr>
<td>3</td>
<td><em>L. acidophilus</em> 2</td>
<td><em>L. crispatus</em></td>
<td>28.6</td>
<td>7.38</td>
</tr>
<tr>
<td>4</td>
<td><em>L. delbrueckii</em></td>
<td><em>L. amylovorus</em></td>
<td>—</td>
<td>&lt;5.30</td>
</tr>
<tr>
<td>5</td>
<td><em>L. fermentum</em></td>
<td><em>L. reuteri</em></td>
<td>—</td>
<td>&lt;5.30</td>
</tr>
<tr>
<td>6</td>
<td>Unknown 1</td>
<td><em>L. amylovorus</em></td>
<td>—</td>
<td>&lt;5.30</td>
</tr>
<tr>
<td>7</td>
<td>Unknown 2</td>
<td><em>L. ruminus</em></td>
<td>—</td>
<td>&lt;5.30</td>
</tr>
<tr>
<td>8</td>
<td>Unknown 3</td>
<td><em>L. amylovorus</em></td>
<td>—</td>
<td>&lt;5.30</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>100</td>
<td>7.93</td>
</tr>
</tbody>
</table>

Lactobacilli were separated as to colony and cell morphotype. One strain of each group was characterized and identified with API 50CHL and the partial 16S rDNA sequencing.

milk in all portions of the pig intestine and feces. In contrast, those of total anaerobes and *Bifidobacteria* were increased by feeding the fermented milk. The number of *Bacteroidaceae* was not affected by feeding the fermented milk. The number of *Lactobacilli* in the small intestine was not affected by feeding the fermented milk, whereas that in the large intestine was increased. The presently used probiotic strain, strain Shirota, was not detected in pigs fed the control diet, while the number of bacteria of this strain was approximately 10<sup>3</sup> to 10<sup>4</sup> cfu/g digesta in the intestine and feces when the fermented milk was administered.

*Flora constitution of Lactobacilli in the feces (Table 3)*

Twenty indigenous fecal *Lactobacilli* randomly isolated from LBS plates were grouped into 3 different colonies and cell morphotypes when the fermented milk was not fed. Thirty *Lactobacilli* were isolated and grouped into 8 types when the fermented milk was fed. The phenotypes of these groups were further identified as *L. brevis* 1 (Group 1) and 2 (Group 2), *L. acidophilus* 2 (Group 3), *L. delbrueckii* (Group 4) and *L. fermentum* (Group 5) according to API 50 CHL. Group 1, 2, 3, 4 and 5 were identified as *L. reuteri, L. reuteri, L. crispatus, L. amylovorus* and *L. reuteri* by partial 16S rDNA sequencing, respectively (sequence similarities between queries and those in database were over 99%, respectively).

Three phenotype groups, i.e. unknown 1 (Group 6), 2 (Group 7) and 3 (Group 8), could not be defined with the API 50 CHL system. On the other hand, Group 6, 7 and 8 were identified as *L. amylovorus, L. ruminus* and *L. amylovorus* by partial 16S rDNA sequencing, respectively (sequence similarities between queries and those in database were over 99%, respectively). The numbers (cfu/g) of Lactobacilli in all the groups were consistently higher when the fermented milk was fed. Moreover, the phenotypic diversity (phenotypic group numbers) of Lactobacilli itself increased from 3 to 8 with the fermented milk supplementation; Group 4, 5, 6, 7 and 8 were now detected. Group 2 was the predominant phenotypic group (68.5% of the total cfu) without fermented milk supplementation, and this group remained
predominant when the fermented milk was fed, but with a reduced proportion (31.6% of the total cfu).

**DISCUSSION**

A large number of bacteria inhabit the large intestine with complex relationships, i.e., symbiosis, and competition for substrates and niches (1, 2, 6). These intestinal bacteria relate to host health, i.e., diarrhea, constipation and infection by pathogens. Probiotics and prebiotics are used for the prevention and treatment of such disorders (7–11). A decrease in the digesta pH due to increased concentrations of lactate and acetate may prevent the growth of pathogenic and potentially pathogenic bacteria (22, 23), which has been recognized as an effect of probiotics and prebiotics. In this experiment, the fecal pH was significantly decreased by fermented milk supplementation. This decrease in pH was associated with decreases in the numbers of Enterobacteriaceae and Enterococci, and with increases in those of Lactobacilli and Bifidobacteria. This pH decrease was due to increases in the acetate and propionate concentrations.

The increases in acetate and propionate are explained by the increases in the numbers of Lactobacilli and Bifidobacteria, the former produce lactate, and the latter acetate and lactate. Since lactate was not detected in large amounts, this acid must have been converted to acetate and propionate by lactate-utilizing bacteria such as Desulfovibrio spp., Propionibacterium spp., Megasphaera spp., Selenomonas spp., and Veillonella spp. (24–26).

It is suggested that the present dose level of fermented milk (130 mL/25 kg body weight), or more precisely the number of live strain Shirota (10^10 bacteria/25 kg body weight), is appropriate for obtaining the colonic fermentation effect.

In the case of humans, the fecal recovery of strain Shirota was 10^5 cfu/g of feces (10^5 cfu excreted per day) when 10^10 cells were administrated daily (19), which was 1,000 times higher than that observed in this experiment. The survival rate of administrated bacteria is affected by gastric acid or bile acids. This rate is also affected by the transit time of digesta. Indigenous bacteria also affect the survival of administrated probiotic strains. The difference in the recovery rates of strain Shirota between the pig and human experiments suggested difference between these potential factors in both animals. L. casei strain Shirota was present at around 10^4 cfu/g digesta in the cecum and the colon. This level seems to be too small to modify the whole fermentation system. We have observed an increase in the population size of indigenous Lactobacilli in the whole large intestine caused by fermented milk: 2.8 times in the cecum and 4.5 times in the colon. In addition to Lactobacilli, Bifidobacteria also showed an increased population size.

The flora constitution of fecal Lactobacilli was significantly changed by feeding the fermented milk, although the predominant species, Group 2, still increased in number and dominated the Lactobacilli flora. Change in the flora constitution suggested that the minor species in the flora under the control conditions might show stimulated growth and become detectable with the fermented milk. Identification of Lactobacilli by API system differed from the result of 16S rDNA sequencing. Although Group 1, 2 and 5 were identified as L. reuteri with 16S rDNA sequencing, their physiological characteristics were different from one another. It was suggested that the phenotypic diversity of Lactobacilli clearly increased with feeding the fermented milk. At present, the mechanisms involved in such stimulation of indigenous Lactobacilli and Bifidobacteria are not clear. The main components of this fermented milk are protein, fat, glucose, sucrose, fructose, lactose and lactate (27). These components except for lactose, however, did not reach the cecum when 2 mL of the fermented milk was orally administrated to rats weighing 250 g. It is suggested in this study that these components will undergo digestion and absorption. On the other hand, lactose could reach the cecum. Ohashi and Umesaki (27) showed that 10% of lactose originally contained in the fermented milk reached the cecum in rats. If lactose flowed into the cecum in our pigs at a similar rate, 100 to 200 mg of lactose could reach the cecum. Although it is not clear whether this amount of lactose can activate colonic fermentation and proliferation of indigenous Lactobacilli and Bifidobacteria, such an amount seems to be negligible for the bulk of cecal contents up to 1 L. (Tsukahara and Ushida, unpublished). As Lactobacillus strains of group 4, 6, 7 and 8 did not ferment lactose, lactose of the fermented milk could not stimulate at least these colonic Lactobacilli that increased the population size. It is likely that factors other than major components of fermented milk may have affected the fermentation and proliferation of indigenous bacteria. There are many reports available on the specific inhibition of the growth of Lactobacilli by bacteriocin, but no report is available on the growth-promoting substances for Lactobacilli produced by probiotic bacteria. Kaneko et al. (28) and Morita et al. (29) reported that some intestinal bacteria produced growth-promoting factors for Bifidobacteria and one substance was identified as quinone. We succeeded in the isolation of a Lactobacillus strain of which in vitro growth was stimulated by fecal aqueous extracts (unpublished). The growth-promoting factors may appear in pig intestine supplemented with fermented milk. Their growth is not promoted simply by biochemical substances, but by some biological conditions such as digesta retention time or digesta pH.

**REFERENCES**