Stimulation of Indigenous Lactobacilli by Fermented Milk Prepared with Probiotic Bacterium, Lactobacillus delbrueckii subsp. bulgaricus Strain 2038, in the Pigs

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Summary The aim of this study was to evaluate the effect of feeding yoghurt, prepared with Lactobacillus delbrueckii subsp. bulgaricus strain 2038, on indigenous lactobacilli in the pig cecum. Three female pigs fistulated at the cecum were fed 250 g of this yoghurt that contained over 10¹¹ colony-forming units of L. delbrueckii subsp. bulgaricus strain 2038 with their daily meal for 2 wk. The relative abundance and the composition of cecal lactobacilli was monitored by analysis of bacterial 16S rDNA with real time PCR and amplified bacterial rDNA restriction analysis using Lactobacillus-group specific primers, respectively, for 2 wk prior to, at the end of 2 wk of and 2 wk after the administration of this yoghurt. The relative abundance of lactobacilli was significantly increased by feeding yoghurt (p<0.01), although the bacterial 16S rDNA matching L. delbrueckii subsp. bulgaricus strain 2038 was not detected by amplified bacterial rDNA restriction analysis during this study. The number of operational taxonomic units (OTUs) detected was increased with feeding of the yoghurt in all pigs. At the same time, the estimated cell number of each OTU was increased with feeding of the yoghurt. It is demonstrated that continuous consumption of the probiotic lactobacilli will stimulate the growth of some indigenous lactobacilli and alter the composition of the lactobacilli.

Key Words yoghurt, probiotic, lactobacillus, L. delbrueckii subsp. bulgaricus strain 2038

The health beneficial effects of probiotic lactic acid bacteria (LAB) such as Lactobacillus casei strain Shirota and L. rhamnosus GG have been reviewed extensively (1, 2). Probiotic LAB may work in the large intestine, which is colonized by numerous indigenous LAB, such as lactobacilli and bifidobacteria. LAB produce the antibacterial substances such as bacteriocin which inhibits the proliferation of other LAB (3). It is reasonable that the probiotic bacteria are also influenced by any antibacterial substance produced by indigenous LAB in the large intestine. Moreover, probiotic bacteria must also compete with indigenous bacteria for substrates and niches to survive (4). However, we previously reported the significant increase in number of the indigenous lactobacilli as a result of an oral dose of probiotic bacterium, L. casei strain Shirota (LeS) (5). The administration of L. plantarum to pigs has also shown to increase the number of indigenous lactobacilli (6). The composition of lactobacilli in humans can be altered by the intake of L. rhamnosus DR20 (7) and L. acidophilus NCFM (8). These studies show that the relationship between probiotic LAB and indigenous LAB is not only competitive, but can also be symbiotic. This positive and negative relationship can affect the level of beneficial effects of probiotic LAB. However, the effect of L. delbrueckii subsp. bulgaricus, used for the preparation of the traditional fermented milk, yoghurt, on the indigenous lactobacilli has not been investigated. In this study, we investigated the effect of yoghurt, prepared with L. delbrueckii subsp. bulgaricus strain 2038 (Ldb) on indigenous lactobacilli in the pig cecum, because this yoghurt has been approved as a food for specified health uses in Japan (9). For this purpose, the cecal digesta was analyzed, because the bacterial fermentative activity is the greatest in the cecum and fermentation pattern was significantly affected by the fermented milk, prepared with Lactobacillus casei strain Shirota (10).

MATERIALS AND METHODS

Animals. Crossbred (Landrace×Large white×Duroc) sows 4 mo old weighing 70.7±2.3 kg (n=3) were used in this experiment. These sows (A, B, and C) were cecostomized to enable digesta sampling. Surgery was conducted according to the operation techniques of Riou et al. (11) and Lefaivre et al. (12). Surgery was completed at least 1.5 mo before the beginning of the experiment. During the experiment, the animals were housed individually in cages. They received 600 g of meals twice a day at 09:00 and 21:00. The meal consisted (g/kg) of cracked maize (670), fish meal (115), alfalfa meal (195), a vitamin mixture (10) and a mineral mixture.
 Indigenous Lactobacilli and Probiotics

The relative abundance of total bacterial DNA and Lactobacillus group-specific DNA in total DNA of cecal bacteria was quantified with the Light Cycler system (Roche Diagnostics, Tokyo, Japan). The FastStart DNA Master SYBR Green I was used for polymerase chain reaction (PCR). The reaction mixture (20 μL) contained 3 mM MgCl₂, 2 μL of the 10×Mastermix, 20 ng of bacterial DNA, and 0.5 μmol/L of each primer. For quantification of total bacterial 16S rDNA, the primers Uni331F (5′-TCTTACGAGCAGTCA-3′) and Uni797R (5′-GGACTACCAGGTATCTATCCTGTT-3′) were used (14). The thermal program consisted of initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 5 s, and the reaction was completed with a final elongation at 72°C for 20 s. For quantification of Lactobacillus group-specific 16S rDNA, the primers S-D-Bact-0011-a-S-17 (5′-AGAGTTTGATYMTGG-GCAGCAGT-3′) and S-G-Lab-0677-a-A-17 (5′-CACCCTCATACATGAG-3′) were used (15). The thermal program consisted of initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 5 s, and the reaction was completed with a final elongation at 72°C for 30 s. The melting curve analyses were performed on the products after the completion of amplifications to determine the specificity of the PCR. Dilutions of the genomic DNA from E. coli JM109 and LcS were used to construct calibration curves. These calibration curves were used for calculation of total bacterial 16S rDNA and Lactobacillus group-specific bacterial 16S rDNA concentration in total cecal digesta DNA, respectively.

Analysis of composition of lactobacilli. Bacterial DNA extracted from the cecal digesta and genomic DNA of Ldb were used for analysis of composition of lactobacilli. The genomic DNA of Ldb was extracted according to Godon et al. (13) after Ldb was cultured in MRS broth (Becton, Dickinson and Company) at 37°C for 12 h. The 16S rDNA was amplified by a PCR with primers S-D-Bact-0011-a-S-17 and S-G-Lab-0677-a-A-17 with rTaq polymerase (TOYOBO). PCR was performed with a temperature program of 4 min at 95°C; 15 cycles of 30 s each at 95°C, 58°C and 72°C; and 7 min at 72°C. The PCR product was subjected to amplified rDNA restriction analysis (ARDRA) using restriction enzymes HaeIII, Rsal and Hhal (TOYOBO) to analyze the composition of lactobacilli after cloning according to Inoue and Ushida (16). The plasmid of 2 or 3 clones from each ARDRA group was subjected to sequencing in a major ARDRA group which consisted of more than 10 clones. Otherwise, the plasmid of one clone was subjected to sequencing. The sequencing was done by the Shimadzu Genomic Research Laboratory (Shimadzu, Kyoto, Japan). Obtained and reference sequences were aligned using the CLUSTAL X program (17). The reference sequences were obtained from the DDBJ. A phylogenetic tree was constructed using the neighbor-joining method by the sequence alignment software (CLUSTAL X). In this study, ARDRA group was regarded as a bacterial operational taxonomic unit (OTU).

Clone numbers in each OTU was regarded as representing the proportion of particular lactobacilli in the cecal population of lactobacilli. Accordingly, population size of particular lactobacilli, hence particular OTU phylogenetically defined as lactobacilli, was estimated by multiplication of the proportion of each OTU by total number of lactobacilli estimated with real-time PCR, hence relative abundance of 16S rRNA gene of lactobacilli. The sum of the estimated cell number of particular OTU phylogenetically defined as lactobacilli was regarded as the total cell number of lactobacilli.

Statistical analysis. The number of cecal bacteria were statistically analyzed by Tukey-PLSD after one-way ANOVA with StatView Ver. 5.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

The relative abundance of total bacterial 16S rDNA.

<table>
<thead>
<tr>
<th></th>
<th>Pr¹</th>
<th>Ad¹</th>
<th>Po¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacteria</td>
<td>10.5±0.2</td>
<td>11.2±0.3</td>
<td>10.8±0.6</td>
</tr>
<tr>
<td>Lactobacillus group</td>
<td>7.23±0.20</td>
<td>7.90±0.05*</td>
<td>7.61±0.28</td>
</tr>
<tr>
<td>Lactobacillus²</td>
<td>6.87±0.05</td>
<td>7.72±0.08*</td>
<td>7.26±0.27</td>
</tr>
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</table>

¹Pr, pre-administration period; Ad, administration period; Po, post-administration period.
²The values were calculated by the sum of the cell number of each OTU belonging to Lactobacillus genera. The cell number of each OTU was estimated by multiplication of the proportion of each OTU by total number of LAB estimated by real-time PCR.
*Significantly different (p<0.01) from Pr. Values were mean±standard deviation (n=3).
which is an estimate of cell number, in cecum was not significantly affected by administration of the yoghurt (Table 1). The relative abundance of *Lactobacillus* group-specific bacterial 16S rDNA was significantly increased in the Ad period (*p*/H11021 < 0.01). The estimated total cell number of lactobacilli also significantly increased in the Ad period (*p*/H11021 < 0.01).

In total, 25 OTUs were identified from cecal digesta of three pigs all over the three experimental periods. The phylogenetic analysis (Fig. 1) of these OTUs showed that 18 OTUs belonged to the cluster constructed by the genus *Lactobacillus*: OTU-1 and -2 associated with *L. delbrueckii* group, and OTU-3, -4 and -5 associated with *L. salivarius*, *L. mucosae* and *L. ruminis*, respectively. Four OTUs (from OTU-15 to OTU-18) associated with *L. amylovorus*. Three other OTUs (OTU-6, -7 and -8) and 6 OTUs (from OTU-9 to OTU-14), respectively formed independent clusters on already-known *Lactobacillus* spp. The OTU matching to Ldb was not detected during this study.

The number of OTU detected was increased with administration of the yoghurt in all pigs (Fig. 2). In pig A, 5 OTUs (OTU-5, -6, -10, -15 and -17) became detectable in Ad, although 3 OTUs (OTU-11, -12 and -14) became undetectable. In pig B, 6 OTUs (OTU-6, -7, -8, -10, -12 and -16) became detectable in Ad. In pig C, 3 OTUs (OTU-2, -12 and -13) became detectable in Ad, although 3 OTUs (OTU-5, -6 and -16) became undetectable in Ad. The cell number of each OTU identified at Ad was increased with the yoghurt (Fig. 2).

**DISCUSSION**

In this study, the relative abundance of *Lactobacillus* group-specific bacterial 16S rDNA was calculated with real-time PCR using the *Lactobacillus* group-specific primers. In the human study, bacterial genera other than *Lactobacillus*, such as *Eubacterium* and *Leuconostoc*, had been detected by the presently used primer set ([5]). The present ARDRA analysis indicated that PCR using this primer set amplified the 16S rDNA of bacteria similar to *Eubacterium* and *Holdmania*. This means that the presently used real-time PCR may overestimate the relative abundance of lactobacilli according to the population sizes of genera *Eubacterium* and *Holdmania*. The former genus is commonly detected in the pig large intestine ([18]). Therefore, the cell number of OTU belong
to lactobacilli was estimated by multiplication of the real-time PCR estimates of Lactobacillus group-specific bacterial 16S rDNA by the proportion of each OTU, and then the sum of these estimated cell numbers was regarded as the total cell number of LAB. However, both estimations gave the same result, a significant increase of lactobacilli in Ad.

Increase in population size of indigenous lactobacilli in pigs as a result of oral administration of live lactobacilli has been reported previously (5, 6). In this experiment, administered Ldb itself seems not to explain the significant increase in the relative abundance of Lactobacillus group-specific 16S rDNA and estimated cell number of lactobacilli in the pig cecum, because the OTU matching to Ldb was not detected during this study. The fecal recovery of Ldb in humans was $10^2$–$10^4$ cfu/g of feces when 100 g of the yoghurt were consumed daily (Kimura et al., unpublished). Although, in our previous study under the similar experimental protocol, orally administered LcS ($10^{11}$ per dose) reached the cecum 6 h after dose (19), the cell number of the administered Ldb which reach the cecum might be small to be detected by ARDRA analysis. The level of Ldb in the cecum may have been the $10^6$ level or less. Nevertheless, the population size of Lactobacillus in the cecum was increased by the yoghurt prepared with Ldb.

Approximately half of OTUs detected in Ad had not been detected in Pr. The lactobacilli belonging to these OTUs were stimulated by yoghurt with Ldb to become detectable by ARDRA, although the predominantly detected OTUs, OTU-1 and OTU-18, in Pr were still predominant lactobacilli in Ad. The increase in population size of L. amylovorus (OTU-18) agrees with our previous report that the fermented milk with LcS stimulated the growth of predominant lactobacilli such as L. amylovorus and L. reuteri (5). Interestingly, OTU-1, the same species as Ldb, increased their population size in Ad. This allogeneic interaction can be explained by the presence of quorum sensing (20). The remaining OTUs newly detected in Ad were OTU-2, -7, -8, -10 and -17. Although OTU-2 and -17 were respectively close to L. delbrueckii subsp. delbrueckii and L. amylovorus, OTU-7, -8 and -10 were separated from already-known lactobacilli. At present, it is difficult to explain the increase in these lactobacilli. In this experiment, pigs received commercially available yoghurt; therefore the starter strain and other fermented milk components besides Ldb could be involved in the effect on indigenous lactobacilli. The nutritive components, such as protein, fat and lactose, included in the yoghurt are believed to be more easily digested and absorbed in the upper gastrointestinal tract than the components including cow’s milk (21, 22). Lactate, which is the end-product in the preparation of yoghurt, does not reach the large intestines, because it is absorbed in the upper gastrointestinal tract (23). Since this yoghurt contained Streptococcus thermophilus as a starter strain, however, the fecal recovery of S. thermophilus in humans was very low ($10^2$–$10^4$ cfu/g of feces) when 100 g of the yoghurt were consumed daily (Kimura et al., unpublished). This cell number is approximately 100 times smaller than that of Ldb. Therefore, the contribution of the nutritive components and S. thermophilus included in the yoghurt to stimulation of indigenous LAB might be a little. The roles of Ldb as a modifier of indigenous lactobacilli in the cecum will be important as in previous reports (5, 24) in which involvement of some growth stimulative substances has been suggested.

Unlike OTUs stimulated by yoghurt, several OTUs becoming undetectable in Ad may have been inhibited by Ldb and/or increased lactobacilli (OTU-1, OTU-18 and others). The predominant species in this study L. delbrueckii subsp. bulgaricus and L. amylovorus, may produce the bacteriocin as described in some strains according to the previous reports (25, 26).

However, the relationship between probiotic lactobacilli and intestinal indigenous lactobacilli is very complex; we must consider not only bacterial ecology but also physiological responses of the intestine, such as absorption and secretion, digesta kinetics and retention time. Digesta kinetics is modified by fermented milk (23) and the cell component of LAB (27). The modification of digesta retention time should affect the bacterial composition, then the fermentation pattern (28). Further in vivo study using appropriate control materials such as unfermented milk will be required for elucidating the specific mechanism of Ldb to stimulate and/or inhibit the growth of indigenous lactobacilli.

In conclusion, the administration of yoghurt prepared with Ldb stimulated the growth of certain indigenous Lactobacillus to modify the composition of cecal lactobacilli in pigs. The result can be extrapolated to
humans, because pig intestinal microbiota is principally similar to that of humans (18, 29).

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REFERENCES