Fluorescent *In Situ* Hybridization Analysis of Cecal Microflora in Rats Simultaneously Administrated *Lactobacillus rhamnosus* KY-3 and Cellobiose

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The cecal microflora of rats coadministered *Lactobacillus rhamnosus* KY-3 (*L. rhamnosus* KY-3) and cellobiose was analyzed by fluorescence *in situ* hybridization. When compared with *L. rhamnosus* KY-3 administration alone, simultaneous administration of *L. rhamnosus* KY-3 and cellobiose led to an increase in the number of lactic acid bacteria (LAB), particularly *L. rhamnosus*, and a significant decrease in the number of Gammaproteobacteria in the cecum. These results indicate that administration of *L. rhamnosus* KY-3 cells passed through the upper digestive tract to the cecum where it is likely that they proliferated through the assimilation of cellobiose.

Keywords: *Lactobacillus rhamnosus*, cellobiose, microflora, FISH, cecum, synbiotics

Introduction

We previously proposed a recycling process for municipal food waste based on polylactic acid production (Sakai *et al.*, 2004). In an attempt to achieve zero emissions for the entire recycling process, we have continued conducting research into reducing and reusing the byproducts of this process. One of the byproducts of the lactic acid produced with our proposed process is the use of *Lactobacillus rhamnosus* KY-3 (*L. rhamnosus* KY-3) as a feed additive.

The combination of pre- and probiotics has been termed “synbiotics” (Roberfroid, 1998) due to their potential for synergy. We investigated *L. rhamnosus* KY-3 for use in synbiotics. Cellobiose was chosen to examine the efficacy of coadministration of probiotic microflora with an indigestible oligosaccharide for use as food. In a study using rats, when *L. rhamnosus* KY-3 (probiotic) was coadministered with cellobiose (prebiotic), concentrations of serum total lipids, triacylglycerol, cholesterol, and phospholipids decreased. However, the decrease in these serum lipids was not observed when *L. rhamnosus* KY-3 was administered alone (Umeki *et al.*, 2004). In order to better understand the mechanisms of such decreases in serum lipids in synbiotic-administered rats, it may be prove useful to analyze changes in the intestinal microflora.

Fluorescence *in situ* hybridization (FISH), which targets specific rRNA sequences, facilitates direct microscopic observation of microorganisms and the gathering of phylogenetic information. We have been using FISH to identify changes in complex microbial flora, such as those seen during open lactic acid fermentation of minced

kitchen refuse (Sakai *et al.*, 2004). Given that FISH is also effective for determining intestinal flora in animals, 16S rDNA oligonucleotide probes specific to the lactic acid bacteria (LAB), *L. plantarum*, *Lactococcus lactis*, and *L. rhamnosus* have been developed (Sakai *et al.*, submitted). Using these probes, we analyzed the cecal microflora in rats receiving *L. rhamnosus* KY-3 and then confirmed the optimal hybridization conditions for detecting LAB and *L. rhamnosus*.

In the present study, we employed the above-mentioned technique in an effort to determine the effects of synbiotic administration (*L. rhamnosus* KY-3 and cellobiose) on the intestinal flora of rats.

Materials and Methods

L. rhamnosus KY-3 was grown to the stationary phase in MRS medium (Atras, 1993) at 37°C for 24 h under anaerobic conditions. Cells collected by centrifugation (10,000×g for 20 min at 4°C) were pelleted and stored at –80°C (without stabilizer) until diet preparation. Cellobiose was obtained from Matsutani Chemical Industry Co., Ltd. (Itami, Japan).

We purchased 3-week-old male Wistar rats from Japan SLC Inc. (Hamamatsu, Japan). Rats were housed in individual cages with stainless steel screen bottoms in a room maintained at 23±1°C and 55±5% relative humidity, with a 12-h light (0800–2000 h) and dark cycle. This study conformed to the guidelines for the care and use of laboratory animals (Notification No. 6 of the Prime Minister's Office, March 27, 1980).

Rats were acclimatized to a stock diet (5L37; Japan SLC Inc., Hamamatsu, Japan) for 3 days. After acclimatization, rats were divided into 4 groups consisting of 6 rats according to body weight. Rats on the control diet were

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fed on a diet containing 20% casein (control diet: casein 200 g/kg; DL-methionine 3 g/kg; sucrose 682 g/kg; soybean oil 70 g/kg; AIN93G mineral mix 35 g/kg; AIN93 G vitamin mix 10 g/kg), supplemented with either *L. rhamnosus* KY-3 (1.7% dry weight; 11% wet weight; 2×10^{13} cells/kg), 10% cellobiose (CEB) or 1.7% *L. rhamnosus* KY-3 and 10% cellobiose (KY-3+CEB). Food and water were provided *ad libitum* for 13 days.

At the end of the 13-day period, each rat was euthanized and the cecum was removed. Cecal contents were analyzed by FISH according to the method of Amann *et al.* (Amann, 1995) under a fluorescence microscope in order to determine the number of all bacteria, most LAB (including lactobacilli and lactococci), *L. rhamnosus* and Gamma-proteobacteria using the 16S rDNA probes, EUB338 (Amann *et al.*, 1990), LAC722 (Sakai *et al.*, 2004), Lrham454 (Sakai *et al.*, submitted) and GAM42a (Manz *et al.*, 1992), respectively. The number of bacterial cells was deter-

mined according to the method of Sakai *et al.* (Sakai *et al.*, 2004).

Data are expressed as means $\pm$ standard error. Data were statistically analyzed by one-way ANOVA. When differences between treatments were significant ($p < 0.05$), Duncan's new multiple range test was performed (Duncan, 1957).

Results and Discussion

Figure 1 shows cell numbers of cecal bacteria, as assessed by FISH. When compared with the control group, the number of most LAB in the KY-3 group and the CEB group was 18- and 4-fold higher, respectively. The numbers of all bacteria and most LAB in the KY-3+CEB group were significantly higher than those in the other groups; the number of most LAB in the KY-3+CEB group was 40-fold higher than that in the control group. When compared with the control group, the number of *L.*

Fig. 1. Populations of bacteria isolated from cecal contents of rats administrated *L. rhamnosus* KY-3 and cellobiose. Rats were fed on 20% casein diets (Control) or experimental diets containing 1.7% dry weight (11% wet weight, 2×10^{13} cells/kg diet) of *L. rhamnosus* KY-3 (KY-3), 10% cellobiose (CEB) or 1.7% *L. rhamnosus* KY-3 and 10% cellobiose (KY-3+CEB). All bacterial cells were enumerated by FISH with the EUB338 probe, while most lactic acid (including lactobacilli and lactococci) bacterial cells, *L. rhamnosus* cells and Gammaproteobacterial cells were enumerated by FISH using the LAC722 (L), Lrham454 and GAM42a probes, respectively. Values are expressed as means $\pm$ standard error ($n=6$), and values not sharing a common letter are significantly different ($p < 0.05$) as analyzed by Duncan's multiple-range test.

rhamnosus in the KY-3 group was significantly higher, with levels in the KY-3+CEB group being the highest (120 times). The relative number of cells stained with Lrham 454 against EUB338 was markedly higher in the KY-3 group (39.0%) and the KY-3+CEB group (47.3%) compared to the control group (2.1%). No corresponding elevation was observed in the CEB group (3.1%). Interestingly, the relative number of *L. rhamnosus* cells in the KY-3+CEB group was slightly elevated as compared to the KY-3 group, implying that the increased numbers of LAB in these two groups could be attributed to *L. rhamnosus* administration, and thus that *L. rhamnosus* KY-3 reaches the intestine without being entirely digested. Furthermore, the number of Lrham454-positive bacterial cells was highest in the KY-3+CEB group, suggesting that *L. rhamnosus* may be able to proliferate by assimilating cellobiose.

The results of the present study showed that the number of Gammaproteobacteria in the CEB and KY-3+CEB groups was significantly lower than the control group. The number of Gammaproteobacteria in the KY-3+CEB group was the lowest among the four groups, and was only one tenth that of the control group. The relative number of cells stained with GAM45a against EUB 338 in the KY-3+CEB group (0.3%) was significantly lower than the control group (14.4%; 1/50) indicating that a decrease in Gammaproteobacteria was involved in the reduced levels of serum lipids. In 1987, Silva *et al.* reported that *L. rhamnosus* GG suppressed the growth of numerous microorganisms, including *Escherichia coli*, by producing a specific antibiotic. Consequently, future research should focus on isolation of such an antibiotic from *L. rhamnosus* KY-3.

The results of the present study corroborate the hypothesis that synbiotic administration is effective in increasing the numbers of most LAB and decreasing the number of the Gammaproteobacteria to which the Gram-negative coliform bacteria belong. Given that this type of improvement in intestinal microflora may be involved in reducing serum lipid levels, *L. rhamnosus* KY-3 may be more useful when administered in the form of a pre-probiotic (synbiotic) with an indigestible oligosaccharide. Use of *L. rhamnosus* KY-3 in pet and cattle feeds appears to be useful for preventing lifestyle-related diseases in

pets and neonatal diarrhea caused by *E. coli* in livestock. Further studies, as well as analyses of microflora by FISH, are thus necessary for investigating the effects of synbiotic LAB administration in pets and livestock.

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References

- Amann, R., Krumholz, L. and Stahl, D.A. (1990). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic and environmental studies in microbiology. *J. Bacteriol.*, **172**, 762–770.
- Amann, R. (1995). In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. *Mol. Microb. Ecol. Manual*, 3.3.61–65., Kluwer Academic Publisher.
- Atlas, R.M. (1993). "Handbook of microbiological medi" ed. by L.C. Parks. CRC Press Inc., Florida.
- Duncan, D.B. (1957). Multiple range tests for correlated and heteroscedastic means. *Biometrics*, **13**, 164–167.
- Manz, W., Amann, R., Ludwig, W., Wagner, M. and Schleifer, K.H. (1992). Phylogenetic oligonucleotide probes for the major subclass of proteobacteria: problems and solutions, **15**, 593–600.
- Roberfroid, M.(1998). Prebiotics and synbiotics: Concepts and nutritional properties. *Br. J. Nutr.*, **80** (suppl. 2), S197–202
- Sakai, K., Taniguchi, M., Miura, S., Ohara, H., Matsumoto, T. and Shirai, Y. (2004). Making plastics from garbage: A novel process for poly-L-lactate production from municipal food waste. *J. Indust. Ecol.*, **7**, 63–74.
- Sakai, K., Mori, M., Fujii, A., Iwami, Y., Chukeyatirote, E. and Shirai, Y. (2004). Fluorescent *in situ* hybridization analysis of open lactic acid fermentation of kitchen refuse using rRNA-targeted oligonucleotide probes. *J. Biosci. Bioeng.*, **98**, 48–56.
- Sakai, K., Oue, K., Umeki, M., Mori, M. and Mochizuki, S. Species-specific FISH analysis of cecal microflora in rats administered with lactic acid bacteria. *World J. Microbiol. Biotechnol.*, (accepted).
- Silva, M., Jacobus, N.V., Deneke, C. and Gorbach, S.L. (1987). Antimicrobial substance from a human *Lactobacillus* strain. *Antimicrob. Agents. Chemother.*, **31**, 1231–1233.
- Umeki, M., Oue, K., Mochizuki, S., Shirai, Y. and Sakai, K. (2004). Effect of *Lactobacillus rhamnosus* KY-3 and cellobiose as synbiotics on lipid metabolism in rats. *J. Nutr. Sci. Vitaminol.*, **50**, 330–334.