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Full Paper

Milk oligosaccharide-mediated cross-feeding between *Enterococcus gallinarum* and lactobacilli in the gut microbiota of infant rats

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We investigated bacteria that have a nutritional symbiotic relationship with respect to milk oligosaccharides in gut microbiota of suckling rats, with specific reference to sialyllactose (SL) degrading *Enterococcus gallinarum*. Our next generation sequencing analysis of the colonic contents of 12-day-old suckling rats revealed that almost half of the bacteria in the microbiota belonged to the Lactobacillaceae family. Major *Lactobacillus* species in the contents were identified as *L. johnsonii*, *L. murinus*, and *L. reuteri*. We then monitored changes in numbers of the above *Lactobacillus* species, *E. gallinarum*, and the bacteria belonging to the family Enterobacteriaceae (i.e., enterobacteria) in the colonic contents of infant rats at 7, 12, 21, 28, and 35 days of age by using real-time PCR assays targeting these bacterial groups. The 7-day-old infant rats had a gut microbiota in which enterobacteria were predominant. Such dominance was replaced by *L. johnsonii* and the concomitant *E. gallinarum* markedly increased in those of 12 and 21 days of ages. During this period, the number of enterobacteria declined dramatically, but that of *L. reuteri* surged dramatically. Our separate *in vitro* experiment showed that supplementation of culture media with SL promoted the growth of *L. johnsonii* and *E. gallinarum*, with marked production of lactic acid. These findings revealed possible milk oligosaccharide-mediated cross-feeding between *E. gallinarum* and *L. johnsonii*, with the former degrading SL to release lactose to be utilized by the latter.

Key words: infant rats, milk oligosaccharide, *Enterococcus gallinarum*, *Lactobacillus johnsonii*, *L. reuteri*

INTRODUCTION

Milk oligosaccharides are resistant to hydrolysis by lactase secreted from the small intestine, and thus most of them reach the large intestine without being absorbed [1, 2]. In the large intestine, most bacteria cannot utilize milk oligosaccharides (MOs), but some limited members of the microbiota can degrade and utilize MOs. In human infants, several species of the genus *Bifidobacterium* are known to specifically degrade human MOs and thus become dominant during the suckling period, resulting in beneficial effects such as intestinal regulation, immunity, and protection against pathobiont infection [3]. For example, bifidobacteria prevent the growth of potentially harmful bacteria by lowering the pH of the infant intestine with organic acids that they produce from MOs [4]. However, little has been reported about such bacteria in gut microbiota of other mammals until recently when Akazawa *et al.* [5] first reported that *Enterococcus gallinarum* isolated from the intestinal contents of suckling rats degraded 3′-sialyllactose (3′-SL) and 6′-sialyllactose (3′-SL), which are major MOs of rats [6]. They [5] also found that the SL degrading *E. gallinarum* preferentially utilized sialic acid rather than lactose released upon degradation and pointed out the possibility that the enterococci "altruistically" degraded SL to cross-feed other members of the gut microbiota of infant rats, as inferred by other researchers' reports on *Bifidobacterium bifidum* with *Bifidobacterium breve* [7, 8]. In the present study, we thus searched for bacteria that become predominant in the intestines of infant rats, with specific reference to SL-mediated cross-feeding, and determined whether such a predominance or surge of specific members of bacteria, including *E. gallinarum*, could be reproduced in vitro by incubating the intestinal contents of infant rats in an SL-supplemented medium. The aim of the study was to help understand how important the indigestible oligosaccharides in breast milk are in the formation of normal gut microbiota in a mammalian host.

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MATERIALS AND METHODS

Collection and preparation of specimens

Fresh intestinal contents (approximately 20–50 mg each) were collected from dissected colon regions of 8 suckling conventional laboratory rats (Jcl:Wistar, CLEA Japan, Inc., Tokyo, Japan) from the same litter that were sacrificed 12 days after birth. In addition, fresh fecal pellets were collected from the mother of the litter. The colonic contents were each suspended in approximately 500 µL of a commercial lyoprotective medium (PreserWell MPR, Funakoshi Co., Ltd., Tokyo, Japan) for use as a primary inoculum (PI) and stored at −80°C in a freezer until being used for next generation sequencing (NGS) analyses and bacteriological in vitro experiments as described below. Meanwhile, fresh contents (approximately 20–50 mg each) were collected from the dissected colon regions of pairs of randomly selected infant rats from 3 other litters, which were sacrificed at 7, 12, 21, 28, and 35 days after birth. It should be noted that the infant rats were allowed to suckle until 21 days of age and weaned thereafter. These colonic contents were found to have pH values ranging from 5.4 to 5.9 and stored at −80°C in a freezer until being used for a subsequent quantitative PCR analysis, as described below. This study was approved by the committee for institutional animal care and use of the Kitasato University Veterinary School (permission No.19-118) and carried out according to its animal experimentation regulations.

Preparation of DNA samples

DNAs were extracted from the stored colonic contents and fecal pellets using the method of Marmur [9], with some modifications. Briefly, 200 µL of the colonic contents or 200 mg of the fecal pellets, 250 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), 50 µL of 10% sodium dodecyl sulfate (Wako Pure Chemical Industries, Osaka, Japan), and glass beads (300 mg, 0.1 mm diameter) with 500 µL of TE-saturated phenol (Nippon Gene, Tokyo, Japan) were shaken vigorously in a FastPrep-24 Instrument (MP Biomedicals SARL, Illkirch, France) for 30 sec at maximum speed. After shaking, the sample was centrifuged (17,120 G) at 4°C for 5 min. After centrifugation, 400 µL of the supernatant was collected in a 1.5 mL microcentrifuge tube (Fukae Kasei, Kobe, Japan), an equal volume of a phenol-chloroform-isoamyl alcohol (25:24:1) mixture (Nippon Gene) was added, and the mixture was centrifuged (17,120 G) at 4°C for 5 min. After centrifugation, 250 µL of the supernatant was collected in 1.5 mL Eppendorf microtubes that were prefilled with 25 µL of 3 M sodium acetate (Wako Pure Chemical Industries, pH 5.4) and 275 µL of isopropanol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The mixture was then allowed to stand at −20°C for 10 min and centrifuged (117,120 G) at 4°C for 5 min. After centrifugation, the supernatant was removed, 500 µL of 70% ethanol (FUJIFILM Wako Pure Chemical Corporation) was added, and the mixture was centrifuged (17,120G) at 4°C for 5 min. The supernatant was then removed, and the DNA pellet obtained was subjected to air-drying for 10–30 min and suspended in 100 µL of TE buffer. The obtained DNA samples thus prepared were stored at −20°C until use.

Next generation sequencing analysis

The DNA samples obtained from the colonic or fecal contents of the 8 suckling rats and their mother were subjected to NGS. Briefly, the V3–V4 regions of the bacterial 16S rRNA gene in the DNA samples were amplified by PCR using an Applied Biosystems SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA), and the amplicons of the 16S V3 and V4 regions were purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA) to remove free primers and primer dimers. PCR reactions were then performed again to add dual index and Illumina sequencing adapters using Nextera XT Index Kit v2 Set A (Illumina, San Diego, CA, USA), and the final library product was purified before quantification using the AMPure XP beads described above. The library was then quantified by measuring the DNA concentration using a Qubit® Assay Kit (Thermo Fisher Scientific). The prepared libraries were subjected to sequence analysis using a MiSeq sequencer (Illumina). The analyzed sequence data were extracted, the number of reads was calculated, and operational taxonomic unit (OTU) clustering and classification at several taxonomic levels, including the phylum, class, family, and genus levels, were performed. An Illumina-curated version of the Greengenes database was used as a taxonomy database for the metagenomics workflow (http://greengenes.secondgenome.com/downloads/database/13_5).

Isolation and identification of lactobacilli

As described below, our meta-16S analysis revealed that the most predominant bacteria in the colonic contents of the suckling rats were lactobacilli. We thus isolated *Lactobacillus* strains from the colonic contents and identified them to the species level. Plates of MRS broth (Difco Laboratories, Detroit, MI, USA), a selective medium for *Lactobacillus*, with 1.5% agar (Wako Pure Chemical Industries) were used for isolation (referred to as MRS agar plates hereafter). Specifically, a loopful each of the thawed PIs of the 8 suckling rats was streaked onto 3 MRS agar plates and incubated anaerobically at 37°C for 24 hr. After incubation, well-isolated single colonies formed on the plates were harvested, and the isolates were inoculated in 1 mL of MRS broth and incubated anaerobically at 37°C for 24 hr. After incubation, genomic DNA was extracted using 200 µL of the culture in the same manner as described above.

The extracted DNAs were then subjected to taxonomic identification to the species level by PCR using primers sets specific for 16S rRNA operons of three species, *Lactobacillus johnsonii*, *L. murinus*, and *L. reuteri*, essentially following the methodologies described elsewhere [10–12] (see Table 1 for details), which are commonly found lactobacilli in the gut microbiota of rodents [13].

Carbohydrate degradation testing of the isolates

As described below, taxonomic identification by PCR assay revealed that the lactobacilli in the colonic contents of the suckling rats mostly comprised *L. johnsonii*, *L. murinus*, and *L. reuteri.* One of each of the strains identified as *L. johnsonii*, *L. murinus*, and *L. reuteri* was thus selected as a representative strain (hereafter referred to as LM1, LJ1, and LR1). These strains and the type strains of the corresponding species (*L. murinus* JCM1717^T, *L. reuteri* JCM1112^T, *L. johnsonii* JCM 2012^T) obtained from the Japan Collection of Microorganisms were subjected to further phenotypic characterization, using a commercial identification kit for *Lactobacillus* species (API 50 CHL Medium, bioMerieux, La Balme-les-Grottes, France), which is designed to identify *Lactobacillus* strains to the species level based on the differences

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Table 1. List of primer sequences and product sizes used for the RT-PCR analysis in this study

Bacterial group	Sequence (5′ to 3′) of primer set used	Amplicon size (nt)	Control DNA from:	Reference
L. murinus	F: AGCTAGTTGGTGGGGTAAAG R: TAGGATTGTCAAAAGATGTC	769	*L. murinus* JCM 1717^T	[10]
L. reuteri	F: ACCGAGAACACCGCGTTATTT R: CATAACTTAACCTAAACAATCAAAGATTGTCT	93	*L. reuteri* JCM 1112^T	[11]
L. johnsonii	F: GAGCTTGCCTAGATGATTTTA R: ACTACCAGGGTATCTAATCC	766	*L. johnsonii* JCM 2012^T	[12]
E. gallinarum	F: TTACTTGCTGATTTTGATTCG R: TGAATTCTTCTTTGAAATCAG	190	*E. gallinarum* JCM 8728^T	[14]
Enterobacteriaceae	F: CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	195	*Escherichia coli* ATCC 43888	[15]
All eubacteria	F: ACTCCTACGGGAGGCAGCAGT R: GTATTACCGCGGCTGCTGGCAC	200	*Escherichia coli* ATCC 43888	[16]

F: forward primer; R: reverse primer.

in their abilities to degrade 49 different carbohydrates.

The abilities of the strains to degrade SL were determined using thin layer chromatography (TLC) plates. Briefly, the strains were inoculated in MRS broth containing 1% SL and incubated anaerobically at 37°C for 24 hr. After incubation, the supernatants (approximately 2 µL each) were each spotted along with 3′-SL and lactose standard solutions (1% wt/vol) onto different lanes of TLC plates (silica gel 60 plate, Merck, Darmstadt, Germany). The plates were developed in ethyl acetate-acetic acid-ethanol-water (12:3:3:2) solvent and then air-dried. Spots were visualized by spraying the plates with *p*-anisaldehyde-acetic acid-sulfuric acid-methanol (1:10:20:200) solution (Tokyo Chemical Industry, Tokyo, Japan) and heating them at 160°C for 2–3 min.

Quantitative PCR (qPCR) assays of the colonic contents of infant rats at different ages

Quantitative PCR (qPCR) assays targeting several groups of bacteria that included the 3 major *Lactobacillus* species, *E. gallinarum*, the family Enterobacteriaceae, and eubacteria (i.e., all bacteria) were performed with a TP700 Thermal Cycler Real Time System Lite (TaKaRa Bio) using the DNA samples prepared from the infants rats at 7, 12, 21, 28, and 35 days of age, essentially following the methodologies described elsewhere [10–12, 14–16] (see Table 1 for details).

In vitro culturing of the infants' colonic contents with and without SL (sialyllactose)

In order to evaluate how SL may influence gut microbiota of the infant rats, an *in vitro* experiment was performed in which PIs of 3 infant rats were incubated with or without SL. Briefly, a modified Gifu anaerobic medium (GAM; with no added sugars; Nissui Pharmaceuticals Co., Ltd, Tokyo, Japan]), from which the agar had been removed using filter paper, was supplemented aseptically with or without a mixture of 3′-SL (Funakoshi) and 6'-SL (Funakoshi) at final concentrations of 1.0% and 0.1%, respectively (hereafter collectively referred to as "SL"). It should be noted that the concentrations of the SLs added to the GAM were comparable to those reported in rat milk [17, 18]. The GAM with or without SL thus prepared (hereafter referred to as GAMSL and GAM, respectively) were inoculated with 10 µL of 3 of the thawed PIs of the 8 infant rats and incubated anaerobically (AnaeroPack Kenki, Mitsubishi Gas Chemical, Tokyo, Japan) at 37°C for 24 hr. After incubation, the pH levels of the cultures were measured, and the cultures were then centrifuged (17,120 G, at 4°C for 5 min) to obtain bacterial pellets and cell-free supernatants, which were subjected to the following assays.

DNAs extracted from the bacterial pellets were subjected to qPCR assays as described above to enumerate bacteria belonging to the 3 *Lactobacillus* species, *E. gallinarum,* and the family Enterobacteriaceae. The supernatants were subjected to HPLC for quantification of the short-chain fatty acids (SCFAs) produced. The HPLC system was equipped with an LC-20AD (Shimadzu) pump, Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA), and Cation H Cartridge (Bio-Rad Laboratories) guard column and maintained at 65°C with a 5 mM sulfuric acid solution as the mobile phase at a flow rate of 0.6 mL/min, in which 10 µL of each filtered spent medium was injected. A differential refractive index detector (RID-10A, Shimadzu) was used for the analysis.

RESULTS

Composition of the gut microbiota of the suckling rats and their mother

The bacterial compositions of the colonic contents of the suckling rats and the fecal pellets of their mother were compared through a meta-16S analysis of the obtained NGS data, and the results are shown in Fig. 1. At the phylum level, the mother's microbiota was almost equally dominated by Firmicutes and Bacteroidetes, while the infants' microbiotas were composed of approximately 60% Firmicutes, 20% Proteobacteria, 10% Bacteroides, and less than 10% Actinobacteria. At the family level, *Lactobacillaceae* and *Enterobacteriaceae* occupied approx. 45% and 20% of the microbiota of the suckling rats, respectively, whereas they composed approx. 10% and less than 1% of that of the mother, respectively. Meanwhile, the family *Enterococcaceae*, which includes the SL-degrading *E. gallinarum*, accounted for approx. 2% of the microbiota of the suckling rats but less than 0.1% of that of the mother.

Taxonomic identities of **Lactobacillus** *strains isolated from colonic contents of the suckling rats and their carbohydrate degrading properties*

Our PCR assays targeting the 16S rRNA sequence specific to the genus *Lactobacillus* showed that 119 out of 144 strains isolated from the colonic contents of the suckling rats belonged to

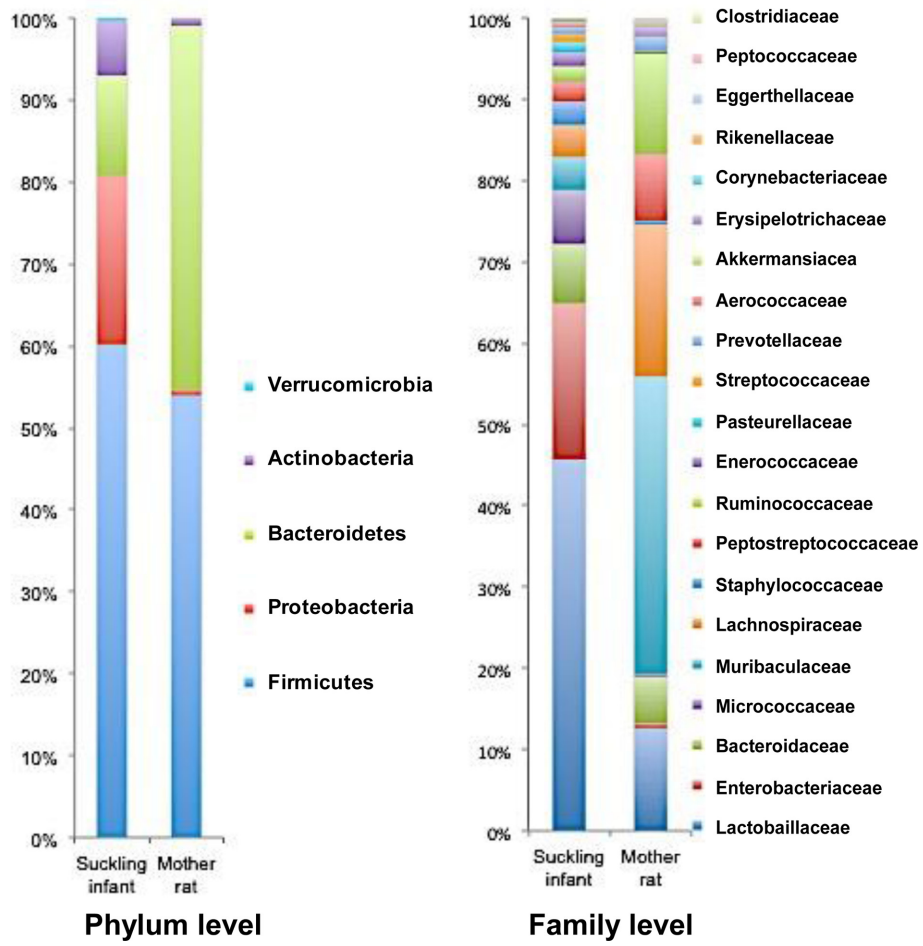

Fig. 1. Comparison of intestinal microflora composition at the phylum and family levels between the suckling rats and their mother by meta-16S analysis.

DNAs extracted from the colonic contents of the 8 suckling rats and their mother were subjected to meta-16S analysis, and the number of reads was calculated. The gut microbiota composition of the mother was based on the number of reads as it was, and that of the suckling rats was based on the average number of reads.

this genus. Subsequent PCR assays of these 119 strains targeting specific 16S rRNA sequences of *L. johnsonii*, *L. murinus*, and *L. reuteri* identified 80 strains as *L. johnsonii,* 27 strains as *L. murinus,* and 12 strains as *L. reuteri*. The degradation profiles of 3 strains (LM1, LJ1, and LR1) randomly selected from the above isolates, representing *L. johnsonii, L. murinus*, and *L. reuteri*, respectively, for 49 different carbohydrates were compared with those of the type strains of the species, and the results are shown in Table 2. Meanwhile, the TLC assays confirmed that none of the above 3 representative isolates or type strains were able to degrade SL (Fig. 2).

Changes in the microbiota of the colonic contents of the infant rats at different ages

The changes in the average numbers of the 3 major *Lactobacillus* species, *E. gallinarum*, and the family *Enterobacteriaceae* (hereafter referred to as enterobacteria) in the colonic contents of the infant rats at different ages are shown in Fig. 3. *E. gallinarum* and *L. johnsonii* increased about 50-fold and more than 10-fold, respectively, from 7 to 12 days of age. From 12 to 21 days of age, the number of *E. gallinarum* decreased to less than 1/100 that at 12 days of age, whereas the number of *L. reuteri* increased from 10^6/g to 10^{11}/g (i.e., a more than 10,000-fold increase). Meanwhile, the numbers of *L. johnsonii and L. murinus* remained at around 10^{12}/g and around 10^{10}/g, respectively, from 7 to 35 days of age. The number of enterobacteria was 10^{12}/g from 7 to 12 days of age but decreased to less than 1/100 that at 12 days of age by 21 days of age and further decreased to less than 1/1,000 at 12 days of age by 28 days. It should be noted that the total bacterial counts remained between 10^{11}/g and 10^{13}/g, slightly higher than that of *L. johnsonii,* from 7 to 35 days of age.

Effects of sialyllactose (SL) on in vitro culturing of the colonic contents

Table 3 shows the results of culturing the colonic contents of the suckling rats at 12 days of age in the modified GAM broth with or without the addition of SL. The numbers of *L. johnsonii* and *L. reuteri* cultured in GAM+SL were more than 10-fold greater than those cultured in GAM, and the differences for both were statistically significant ($p<0.05$ and $p<0.01$, respectively, by paired Student's t-tests). The numbers of *L. murinus* and *E. gallinarum* were also 2- to 3-fold greater in GAM+SL than

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Table 2. Carbohydrate fermentation profiles of the 3 *Lactobacillus* isolates from the colonic contents of the suckling infant laboratory rats, representing *L. murinus*, *L. johnsonii*, and *L. reuteri*, and the type strains

Carbohydrates tested

Strain ID No.	Taxonic Identity based on 16S rRNA	Glycerol	Erythritol	D-Arabinose	L-Arabinose	Ribose	D-Xylose	L-Xylose	Adonitol	β-Methyl-xyloside	Galactose	D-Glucose	D-Fructose	D-Mannose
JCM1717^{T}	*Lactobacillus murinus*	-	-	-	-	-	-	-	-	-	+	+	+	+
LM1	*Lactobacillus murinus*	-	-	-	-	-	-	-	-	-	+	+	+	+
JCM2012^{T}	*Lactobacillus johnsonii*	-	-	-	-	-	-	-	-	-	+	+	+	+
LJ1	*Lactobacillus johnsonii*	-	-	-	-	-	-	-	-	-	+	+	+	+
JCM1112^{T}	*Lactobacillus reuteri*	-	-	-	+	+	-	-	-	-	+	+	-	-
LR1	*Lactobacillus reuteri*	-	-	-	+	+	+	-	-	-	+	+	±	-

Strain ID No.	Taxonic Identity based on 16S rRNA	L-Sorbose	Rhamnose	Dulcitol	Inositol	Mannitol	Sorbitol	α Methyl-D-mannoside	α Methyl-D-glucoside	N Acetyl glucosamine	Amygdaline	Arbutine	Esculine
JCM1717^{T}	*Lactobacillus murinus*	-	-	-	-	±	-	-	-	+	-	±	+
LM1	*Lactobacillus murinus*	-	-	-	-	-	-	-	-	+	-	-	+
JCM2012^{T}	*Lactobacillus johnsonii*	-	-	-	-	-	-	±	±	+	+	+	+
LJ1	*Lactobacillus johnsonii*	-	-	-	-	-	-	±	±	+	±	±	+
JCM1112^{T}	*Lactobacillus reuteri*	-	-	-	-	-	-	-	-	-	-	-	-
LR1	*Lactobacillus reuteri*	-	-	-	-	-	-	-	-	-	-	-	-

Strain ID No.	Taxonic Identity based on 16S rRNA	Salicine	Cellobiose	Maltose	Lactose	Melibiose	Saccharose	Trehalose	Inulin	Melezitose	D-Raffinose	Amidon	Glycogene
JCM1717^{T}	*Lactobacillus murinus*	+	+	+	+	+	+	-	-	-	+	-	-
LM1	*Lactobacillus murinus*	+	+	+	+	+	+	-	-	-	+	-	-
JCM2012^{T}	*Lactobacillus johnsonii*	+	+	+	+	+	+	±	±	±	+	+	±
LJ1	*Lactobacillus johnsonii*	+	+	±	+	±	+	+	-	-	-	-	-
JCM1112^{T}	*Lactobacillus reuteri*	-	-	+	+	+	+	-	-	-	+	-	-
LR1	*Lactobacillus reuteri*	-	-	+	-	+	+	-	-	-	+	-	-

Strain ID No.	Taxonic Identity based on 16S rRNA	Xylitol	β Gentiobiose	D-Turanose	D-Lyxose	D-Tagatose	D-Fucose	L-Fucose	D-Arabitol	L-Arabitol	Gluconate	2 keto-gluconate	5 keto-gluconate
JCM1717^{T}	*Lactobacillus murinus*	-	±	-	-	-	-	-	-	-	-	-	-
LM1	*Lactobacillus murinus*	-	-	-	-	-	-	-	-	-	-	-	-
JCM2012^{T}	*Lactobacillus johnsonii*	±	+	-	-	+	-	-	-	-	-	-	-
LJ1	*Lactobacillus johnsonii*	-	+	-	-	-	-	-	-	-	-	-	-
JCM1112^{T}	*Lactobacillus reuteri*	-	-	-	-	-	-	-	-	-	+	-	-
LR1	*Lactobacillus reuteri*	-	-	-	-	-	-	-	-	-	±	-	-

Results: +, positive; ±, weakly positive; −, negative.

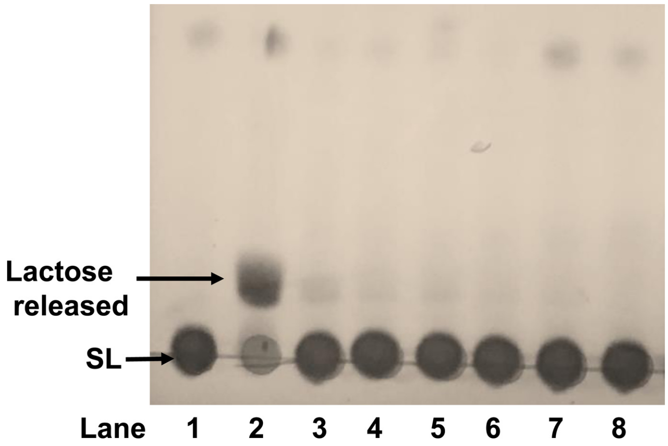

Fig. 2. TLC of spent cultures of GAM+SL inoculated with the 3 representative lactobacilli isolates from the colonic contents of the suckling rats.

Lanes: 1, PSB added with 3′-SL (Funakoshi) and 6’-SL (Funakoshi) at a final concentration of 1.0% and 0.1%, respectively; 2, GAM+SL incubated with *E. gallinarum* AH4 (as a positive control for 3′-SL degradation); 3, GAM+SL incubated with *L. johnsonii* LJ1; 4, GAM+SL incubated with *L. murinus* LM1; 5, GAM+1% SL incubated with *L. reuteri* LR1; 6, GAM+1%SL incubated with *L. johnsonii* JCM 2012^{T}; 7, GAM+SL incubated with *L. murinus* JCM 1717^{T}; 8, GAM+SL incubated with *L. reuteri* JCM 1112T.

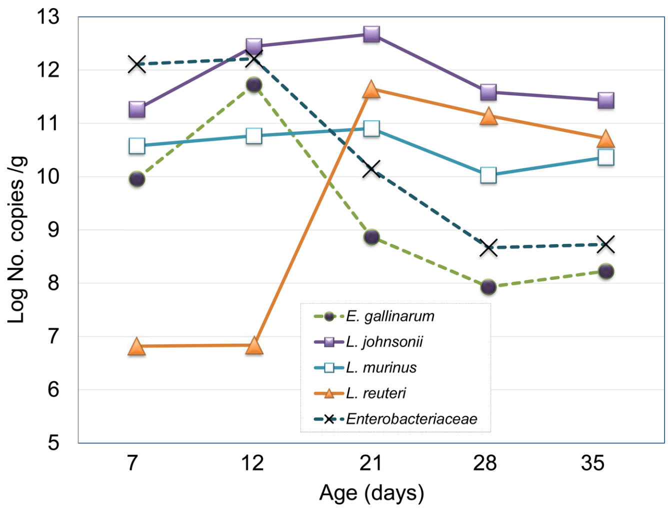

Fig. 3. Bacterial counts for each major bacterial group in the colonic contents of the infant rats at different ages.

Each dot indicates the logarithm of the mean number (copy number) of each bacteria bacterial group in the colonic contents of 3 infant rats. It should be noted that suckling was stopped at 21 days of age.

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in GAM, but the differences were not statistically significant. The numbers of enterobacteria and total eubacteria grown in GAM+SL and GAM remained comparable.

Our TLC assays for the spent media confirmed that sizable amounts of SL were consumed and that lactose moieties were correspondingly liberated in all SL supplemented wells (Fig. 4). The results of the HPLC assays for the spent media (Table 4) showed that the amount of lactic acid produced in the GAM+SL increased to more than 3-fold that in GAM, which was statistically significant ($p<0.01$, paired Student's t-test), while the amounts of acetic acid, succinic acid, and propionic acid were comparable between GAM and GAM+SL. The total amount of SCFA was thus significantly greater ($p<0.05$, paired Student's t-test) in GAM+SL than in GAM, with the average pH values of the spent media in the former and latter cases being 6.1 and 7.0, respectively.

DISCUSSION

In the present study, we revealed that the major bacterial group of the colonic microbiota of the suckling rats at 12 days of age belonged to the family *Lactobacillaceae*, comprising mostly *L. johnsonii*, *L. murinus*, and *L. reuteri*. Meanwhile, our qPCR assays for the colonic contents of the infant rants at different ages showed that *L. johnsonii* increased markedly from 7 to 12 days after birth and that the SL-degrading *E. gallinarum* increased almost in parallel. This concerted increase was reproduced in our *in vitro* experiments, in which *L. johnsonii* and *E. gallinarum* grew well in SL-supplemented medium. *L. johnsonii* is widely distributed as a commensal bacterium in the intestines of mammals, including rats [19]. The strain of *L. johnsonii* isolated in this study and its type strain were found to be capable of utilizing lactose but not SL. *E. gallinarum* is a common inhabitant of the intestines in both humans and animals [20]. Recently, Akazawa *et al.* [5] demonstrated in an *in vitro* experiment that *E. gallinarum* isolated from suckling rats was capable of degrading SL but that the bacterium preferentially utilized sialic acids rather than lactose released upon degradation. From this evidence, they speculated that lactose moieties released by the *E. gallinarum* would promote the growth of other concomitant bacteria, as demonstrated by other researchers [7, 8] with bifidobacteria of human infant origin, the studies of which showed that *B. bifidum* degraded human MOs to "altruistically" feed lactose to *B. breve* upon degradation. Our findings in the present study thus indicate that *E. gallinarum* and *L. johnsonii* may have a "rat version" of such cross-feeding.

Meanwhile, the number of enterobacteria exceeded that of lactobacilli by more than 5-fold in infant rats at 7 day of age. Since many members of enterobacteria are known to be vigorous lactose utilizers, they would have been prime beneficiaries of the lactose released by the concomitant *E. gallinarum* upon SL degradation. This was, however, not the case, as they did not increase but were instead surpassed by *L. johnsonii* in the guts of the infant rats at 12 days of age. This evidence suggests that the lactose moieties are utilized preferentially by *L. johnsonii* rather than other concomitant bacteria. Some *Lactobacillus* species have been reported to adhere to other symbiotic microorganisms to form biofilms and establish co-nutritional relationships [21]. *E. gallinarum* and *L. johnsonii* may thus adhere to each other to help establish a co-nutritional relationship. This will be verified in our next study, which is currently in progress.

Table 3. Bacterial counts of the major bacterial groups after 24 hr of anaerobic culturing of the colonic contents of the suckling rats in the medium supplemented with and without sialyllactose (SL)

Bacterial group	Log No. copies /mL[1)] after incubation in:	
	GAM+SL	GAM
L. johnsonii	10.62 (0.14)*	9.12 (0.40)
L. murinus	9.51 (0.59)	9.10 (0.61)
L. reuteri	8.42 (0.16)**	7.62 (0.22)
E. gallinarum	8.98 (0.32)	8.51 (0.64)
Enterobacteriaceae	11.02 (0.15)	10.97 (0.08)
All eubacteria	10.98 (0.08)	11.08 (0.1)

[1)] Mean of logarithmic number (copy number) of each bacterial group in the cultured colonic contents of 3 suckling rats, with standard deviation in parentheses.
*$p<0.05$ by paired Student's t-test. **$p<0.01$ by paired Student's t-test.

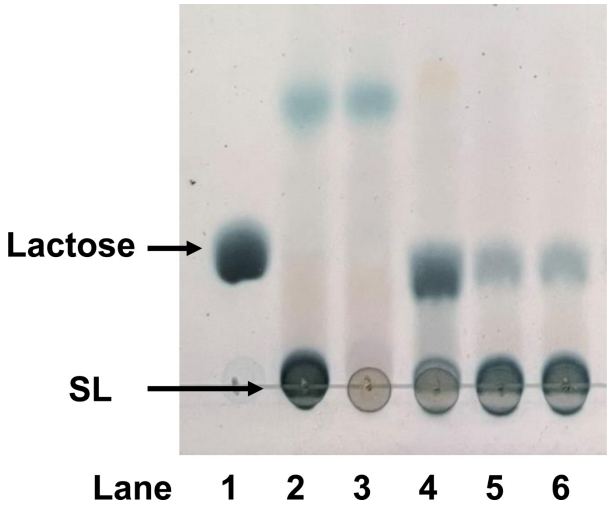

Fig. 4. TLC of spent cultures of GAM+SL inoculated with suckling rat colonic contents.

Lanes: 1, 0.5% lactose in PBS; 2, GAM+SL before incubation; 3, GAM before incubation; 4, GAM+SL incubated with the colonic contents of suckling rat No. 1; 5, GAM+SL incubated with the colonic contents of suckling rat No. 5; 6, GAM+SL incubated with the colonic contents of suckling rat No. 8. Arrows indicate spots for lactose and SL.

Table 4. Amounts of short-chain fatty acids produced after 24 hr of anaerobic culturing of the colon contents of the suckling rats in the medium supplemented with and without sialyllactose (SL)

SCFA	mM[1)] produced after incubation	
	GAM+SL	GAM
Acetate	32.82 (11.59)	28.99 (1.79)
Propionate	4.67 (1.20)	4.77 (0.14)
Butyrate	<1.0	<1.0
Lactate	10.37 (2.11)**	2.97 (1.13)
Succinate	10.58 (3.10)	7.19 (0.30)
Total	58.44 (11.56)*	43.92 (2.20)

[1)] Mean concentration (mM) of each SCFA in the cultured colonic contents of 3 suckling rats, with standard deviation in parentheses.
*$p<0.05$ by paired Student's t-test. **$p<0.01$ by paired Student's t-test.

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In our *in vitro* experiments, the production of lactic acid after incubation of the colonic contents of the infant rats with SL was 3-folds higher than that of those without SL, and the average pH value was almost one magnitude lower in the former case than in the latter case. Most, if not all, lactobacilli grow optimally in a slightly acidic environment [22], whereas the growth of enterobacteria is mostly inhibited in such an environment [23]. The lactic acid produced by *L. johnsonii* from the lactose liberated by *E. gallinarum* upon its degradation of SL might have decreased the pH level and inhibited the growth of enterobacteria in the medium. If so, this could be yet another "rat version" of what takes place in human infants, as described by Mitsuoka [4], who reported that bifidobacteria proliferate on maternal milk oligosaccharides and lower the pH in the colonic environment, thereby suppressing the growth of potentially pathogenic enterobacteria and maintaining a healthy state of the host's intestinal environment. This was, however, not confirmed in our in vitro experiment, which showed that the growth of enterobacteria in the colonic cultures was comparable between GAM+SL and GAM. This inconsistency may be ascribed to the fact that the culture medium used did not contain lactose, which is a major carbohydrate in rat maternal milk [18]. It has been long reported that lactose entering the lower part of the intestine of rats is subjected to microbial fermentation there that produces mainly lactic acid and short-chain fatty acids, thereby lowering the pH [24]. In fact, the pH values of the colonic contents used in the present study ranged from 5.4 to 5.9, whereas those of the spent media in our *in vitro* experiment ranged from 6.0 to 7.0, which almost coincided with the optimal pH range for growth of *Escherichia coli* for instance [25].

It may be of particular interest that the number of *L. reuteri* in the gut microbiota of the infant rats at 21 days of age increased to more than 10,000-fold that of those at 12 days of age. This seems to have been reproduced in our in vitro experiment, in which we observed a marked increase in *L. reuteri* along with *L. johnsonii* in the SL-supplemented medium. Unlike the type strains of *L. reuteri* and *L. johnsonii*, *L. reuteri* LR1 isolated from the infant rats was unable to utilize lactose. Although highly speculative, if *L. reuteri* with such a trait was prevalent in the guts of the suckling rats, the growth of *L. reuteri* might be promoted by some other causes than the lactose supplied by the concomitant *E. gallinarum*. One of the candidate causes is linked to the fact that some strains of *L. reuteri* are able to produce a broad-spectrum antimicrobial substance called "reuterin" that suppresses the growth of non-lactobacilli bacteria, such as enterobacteria and enterococci [26]. If this is the case, the "rise" in *L. reuteri* may be yet another contributing factor to the "fall" in the potentially pathogenic bacteria in the gut microbiota of infant rats. As a supplementary note, *L. reuteri* has been recently redefined as *Limosilactobacillus reuteri* [27].

The rodent *L. reuteri* is known to utilize D-xylose, one of the constituent sugars of hemicellulose in cereal grains such as corn and wheat bran consumed by adult rats [28]. *L. reuteri* is also known to promote the production of butyric acid, which is important for the health of intestinal epithelial cells, through symbiosis with butyrate-producing bacteria [29]. In the present study, the *L. reuteri* strain isolated from the rats was confirmed to utilize D-xylose. In humans, bifidobacteria proliferated by milk oligosaccharides during infancy are known to supply monosaccharides and disaccharides to butyrate-producing bacteria to aid in the transition to a normal adult-type gut microbiota [30]. It is thus probable that *L. reuteri* plays a similar role in infant rats with specific reference to butyrate production. Although no butyrate production was detected in our *in vitro* experiment, the above hypotheses can be verified by conducting additional *in vitro* experiments. For example, we could conduct an experiment in which the colonic contents of infant rats are cultured in GAM supplemented with plant-derived substrates (e.g., hemicellulose) as a model of the post-weaning period. Such attempts would further our understanding of the roles of a variety of bacterial members and their metabolites and the sequences of their events in establishing healthy gut environments after birth in many mammalian species, including humans.

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