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Full Paper

Isolation and identification of milk oligosaccharide-degrading bacteria from the intestinal contents of suckling rats

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We report the isolation of bacteria capable of degrading milk oligosaccharides from suckling infant rats. The bacteria were successfully isolated via a selective enrichment method, in which the serially diluted intestinal contents of infant rats were individually incubated in an enrichment medium containing 3′-sialyllactose (3′-SL), followed by the isolation of candidate strains from streaked agar plates and selection of 3′-SL-degrading strains using thin-layer chromatography. Subsequent genomic and phenotypic analyses identified all strains as *Enterococcus gallinarum*. The strains were capable of degrading both 3′-SL and 6′-SL, which was not observed with the type strain of *E. gallinarum* used as a reference. Furthermore, a time-course study combining high-performance anion-exchange chromatography with pulsed amperometric detection revealed that the representative strain AH4 degraded 3′-SL completely to yield an equimolar amount of lactose and an approximately one-fourth equimolar amount of sialic acid after 24 hr of anaerobic incubation. These findings point to a possibility that the enterococci degrade rat milk oligosaccharides to "cross-feed" their degradants to other members of concomitant bacteria in the gut of the infant rat.

Key words: suckling rats, sialylated lactose, sialic acid (*N*-acetylneuraminic acid), serial dilution selective enrichment, *Enterococcus gallinarum*

INTRODUCTION

One of the major evolutional features for mammalian species is that they produce milk to feed their offspring [1, 2]. Their milk contains all of the essential nutrients (i.e., carbohydrates, fat), antibodies, and other factors important for the growth and development of their infants [3, 4]. In human milk, lactose, which is composed of glucose and galactose bonded by a β1-4 glycosidic linkage, constitutes 80% of the carbohydrates, whereas more than 200 species of oligosaccharides, which are collectively termed human milk oligosaccharides (HMOs) and are created via conjugations of glucose, galactose, *N*-acetylglucosamine, fucose, and sialic acid, account for the remaining content [4, 5]. Unlike lactose, HMOs are not readily digested by host intestinal lactase, but they can be degraded and utilized by some particular members of the gastrointestinal microbiome with specific reference to human residential bifidobacteria (e.g., *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium bifidum*) [6–8]. Breastfed human infants are thus able to develop a bifidobacteria-dominant microbiome in their intestine [9–11], which appears to play important roles in preventing colonization by pathogenic bacteria and complementing the proper development of the infant immune system [12–14].

Meanwhile, little research has examined whether any other mammalian species have such milk oligosaccharide-mediated symbiotic relationships between infants and their gastrointestinal microbiome. Rodents, for example, feed their infants milk containing two oligosaccharides, namely 3′-sialyllactose (3′-SL) and 6′-SL, which consist of the monosaccharide *N*-acetylneuraminic acid (NANA) linked to the galactosyl residue of lactose at the third and sixth positions, respectively [15]. 3′-SL is 10-fold more abundant than 6′-SL in rat milk oligosaccharides [16], the total concentration of which was reported to range from 7.1 mM to 25.9 mM [17]. To the best of our knowledge, however, no study has been conducted to elucidate whether any bacteria in the gut microbiota of infant rats are capable of degrading milk

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oligosaccharides. We herein report the isolation of bacteria from the intestinal contents of infant rats capable of degrading both 3′-SL and 6′-SL and their taxonomic identities along with their biochemical properties.

MATERIALS AND METHODS

Preparation of culture inocula of suckling rats

Fresh intestinal contents (approximately 20–50 mg each) were collected from the dissected colon regions of eight sucking conventional laboratory rats (Jcl:Wistar, CLEA Japan, Inc., Tokyo, Japan) of the same litter that were sacrificed 12 days after birth. Each intestinal sample was 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 use.

Preliminary screening for bacterial cultures exhibiting 3′-SL degradation

The frozen PIs were thawed at room temperature, and then 2-fold serial dilutions of each inoculum starting at 1:100 and ending at 1:1 × 2^{30} (approximately 1 trillion) were made in v-bottom 96-well plates (pre-sterilized), with each well containing 125 µL of modified Gifu anaerobic medium (GAM; with no added sugars; Nissui Pharmaceuticals Co., Ltd, Tokyo, Japan) supplemented with a filter-sterilized solution of 3′-SL (Funakoshi) at a final concentration of 1%. This concentration was comparable to the concentration of 3′-SL in rat milk during lactation, as reported elsewhere [16, 17]. Eight sets (from the eight rats) of the serially diluted inocula were then incubated at 37°C for 48 hr under anaerobic conditions using AnaeroPack (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan). After incubation, the plates were centrifuged to separate bacterial pellets and supernatants. After centrifugation, the supernatants were then transferred to new sets of 96-well plates for subsequent thin-layer chromatography (TLC) to identify spent medium with degraded 3′-SL. Briefly, 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 at 160°C for 2–3 min. Meanwhile, the bacterial pellet at the bottom of each well was suspended with approximately 100 µL of lyoprotective medium (Funakoshi), and the prepared bacterial suspensions were stored at −80°C in a freezer until use.

Isolation and identification of 3′-SL-degrading bacteria

The bacterial suspension of the well with the highest intestinal content dilution exhibiting apparent 3′-SL degradation by TLC was selected for each of the eight suckling rats to isolate 3′-SL-degrading bacteria as follows. First, the frozen bacterial suspensions were thawed at room temperature, and then each suspension was streaked on GAM agar plates using platinum loops. The agar plates were then anaerobically incubated at 37°C for 48 hr using AnaeroPack. After incubation, 5–10 well-separated colonies on each plate were randomly selected and screened for 3′-SL degradation using our aforementioned methodology.

Three of the 3′-SL–degrading bacterial isolates, designated as strains AH1, AH4, and AH6, were randomly selected to determine taxonomic identities via 16S rRNA sequencing. The whole-genome DNA of each strain was prepared essentially following the procedure of Marmur[18]. Each DNA preparation was then used to obtain a fragment of continuous 16S rRNA operon (approximately 1440 bp) by PCR using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) [19]. PCR products were then purified using a NucleoSpin Extract II Kit (Macherey-Nagel, Duren, Germany). Finally, sequencing of the PCR products was performed using a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK) and Applied Biosystems 3100xl Genetic Analyzer (Applied Biosystems, Darmstadt, Germany).

It has long been claimed that the 16S rDNA approach fails to identify enterococcal isolates to the species level because many of them, including *Enterococcus gallinarum*, share nearly or more than 99% identity in their 16S rRNA gene sequences [20]. We thus performed an additional PCR assay designed to target an *E. gallinarum*-specific sequence of the housekeeping gene *sodA* in the strains and the *E. gallinarum* type strain JCM8728^{T}, as a positive control, using the primer set GA1 (5′-TTACTTGCTGAT TTTGATTCG-3′) and GA2 (5′-TGAATTCTTCTTTGA AATCAG-3′), as described by Jackson *et al.* [21].

Morphological and biochemical characterization

Four randomly selected strains and *E. gallinarum* JCM8728^{T} were subjected to Gram staining, and their cell shapes and formation were observed under an oil microscope. Meanwhile, the biochemical characteristics of these strains were determined using the Rapid ID 32 Strep system (bioMérieux, Lyon, France). In addition, the abilities of these strains to degrade 6′-SL (16 mM, Funakoshi) and lactose (3 mM, Wako Pure Chemical, Osaka, Japan), as well as 3′-SL (16 mM, Funakoshi), were examined by TLC using the aforementioned protocol.

Time-course study of 3′-SL degradation using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

The time course of the degradation of 3′-SL was examined using HPAEC-PAD to more quantitatively assess 3′-SL degradation by the strains. Briefly, freshly harvested bacterial cells of AH4 that had grown anaerobically to the exponential phase in GAM (with no added sugar) were inoculated at a final concentration of approximately 1.0 × 10^{7} cfu/mL into the wells of 96-well plates (pre-sterilized), each of which contained 200 µL of GAM supplemented with 3′-SL (final conc. 1%: approximately 16 mM). The plates were then incubated anaerobically at 37°C for up to 48 hr, and cultures were removed from a set of the three wells (for triplicate testing) at 3, 6, 9, 12, 15, 18, 24, 30, 36, and 48 hr and centrifuged to obtain cell-free spent media. The spent media were then subjected to subsequent HPAEC-PAD analysis as follows. The analytes were separated on a Dionex ICS-3000 system equipped with a CarboPac PA-1 column (Fisher Scientific, Waltham, MA, USA) maintained at 30°C. Elution was performed under constant flow (0.25 mL/min) with a linear gradient of 0–330 mM sodium acetate in 125 mM NaOH at 30°C for 20 min. The standard curve was constructed from known concentrations

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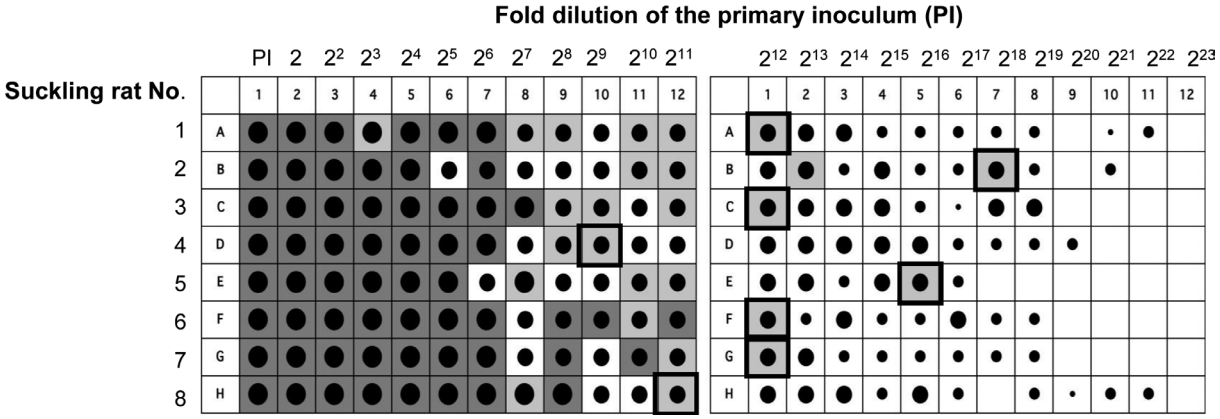

Fig. 1. Schematic drawing of growth and 3′-sialyllactose (3′-SL) degradation in 96-well plates containing a 3′-SL-supplemented medium inoculated with the intestinal contents of eight rats at various concentrations.

Differing sizes of black spots and color tones in brackets represent the scale of bacterial growth and 3′-SL degradation in the corresponding wells of the plates, respectively.

Bold-bracketed wells indicate the cultures that were streaked on agar plates to isolate bacterial strains.

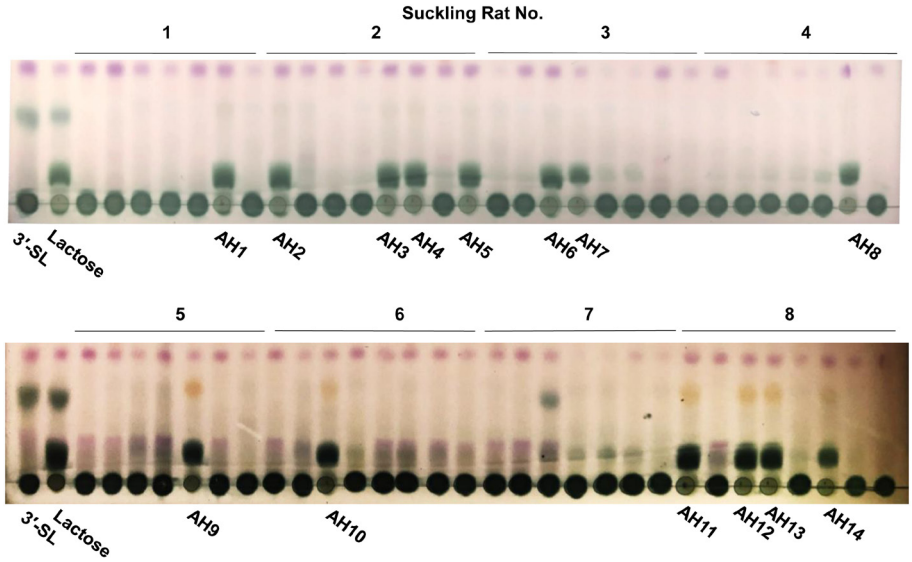

Fig. 2. Thin-layer chromatography of 3′-sialyllactose (3′-SL)-degrading activities for 60 bacterial isolates from eight suckling rats.

3′-SL and lactose were spotted as standard saccharides on lanes 1 and 2 of the chromatograph. AH1–AH14 are the strains that degraded 3′-SL.

of the sugars. The samples were kept at approximately 10°C in the autosampler.

RESULTS

Isolation of 3'-SL-degrading bacteria

In our dilution enrichment analysis, apparent bacterial growth was observed in wells that had been inoculated with the suckling rats' intestinal contents diluted up to 2^{18}- to 2^{22}-fold, whereas appreciable 3′-SL degradation was observed following dilution up to 2^{9}- to 2^{18}-fold (Fig. 1). A total of 60 colonies were isolated from the GAM agar plates that had been streaked with the cultures from the wells with the highest dilution exhibiting sizable 3′-SL degradation. Subsequent TLC analysis of the isolates illustrated that 14 isolates, designated strains AH1–AH14, degraded 3′-SL to release lactose molecules (Fig. 2).

Genomic identification of the 3'-SL-degrading isolates

The 16S rRNA gene sequences of the PCR products of the 3′-SL-degrading strains (data not shown) exhibited more than 99% sequence identity to *E. gallinarum* in a similarity search analysis using the BLAST algorithm in the NCBI database (https://blast.ncbi.nlm.nih.gov). Subsequent PCR analysis using primer pairs targeting the *E. gallinarum*-specific *sodA* sequence confirmed that the 14 isolates were *E. gallinarum* (data not shown).

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Table 1. Physiological characteristics of 3′-sialyllactose-degrading isolates AH1–AH4 from suckling rats and the *Enterococcus gallinarum* type strain for comparison, as determined using the Rapid ID 32 Strep system

	Biochemical characteristics as determined by Api Rapid ID32 Strep system															
Strain No.	ADH	βGLU	βGAR	βGUR	αGAL	PAL	RIB	MAN	SOR	LAC	TRE	RAF	SAC	LARA	DARL	CDEX
AH1	+	+	+	-	+	-	+	-	-	+	+	+	+	+	-	+
AH3	+	+	+	-	+	-	+	-	-	+	+	+	+	+	-	+
AH4	+	+	-	-	+	-	+	-	-	+	+	+	+	+	-	+
AH5	+	+	+	-	+	-	+	-	-	+	+	+	+	+	-	+
Enterococcus gallinarum JCM8728^T	+	+	-	+	+	-	+	+	-	+	+	+	+	+	-	+

	Biochemical characteristics as determined by Api Rapid ID32 Strep system															
Strain No.	VP	APPA	βGAL	PYRA	βNAG	GTA	HIP	GLYG	PUL	MAL	MEL	MLZ	MBDG	TAG	βMAN	URE
AH1	+	-	+	+	+	+	+	-	-	+	+	-	+	+	+	-
AH3	+	-	+	+	+	+	+	-	-	+	+	-	+	+	+	-
AH4	+	-	+	+	+	+	+	-	-	+	+	-	+	+	+	-
AH5	+	-	+	+	+	+	+	-	-	+	-	-	+	+	+	-
Enterococcus gallinarum JCM8728^T	+	-	+	+	+	+	+	-	-	+	+	-	+	+	+	-

ADH: arginine dihydrolase; βGLU: β-glucosidase; βGAR: β-galactosidase; βGUR: β-glucuronidase; αGAL: α-galactosidase; PAL: alkaline phosphatase; RIB, MAN, SOR, LAC, TRE, RAF, SAC, LARA, DARL, and CDEX, fermentation of ribose, mannitol, sorbitol, lactose, trehalose, raffinose, sucrose, L-arabinose, d-arabitol, and cyclodextrin, respectively; VP: Voges-Proskauer test; APPA: activity of alanine-phenylalanine-proline arylamidase; βGAL: β-galactosidase; PYRA: pyrrolidonyl arylamidase; βNAG: N-acetyl-β-glucosaminidase; GTA: glycyl-tryptophan arylamidase; HIP: hydrolysis of hippurate; GLYG, PUL, MAL, MEL, MLZ, MBDG, and TAG, fermentation of glycogen, pullulan, maltose, melibiose, melezitose, methyl-d-glucopyranoside, and tagatose, respectively; βMAN: β-mannosidase; URE: urease.

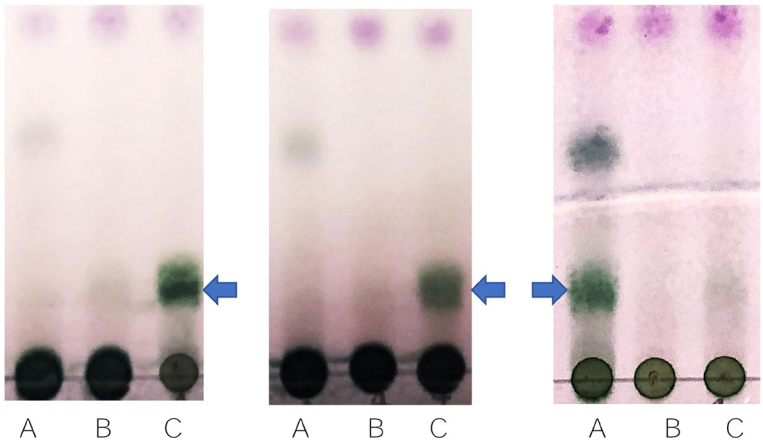

Fig. 3. Thin-layer chromatography profiles of 3′-sialyllactose, 6′-SL, and lactose utilization by the type strain of *Enterococcus gallinarum* and strain AH4.

Left TLC (3′-SL), middle TLC (6′-SL), and right TLC (lactose). Lane A, dissolved in the medium spotted as standards; lane B, the spent medium of *E. gallinarum* JCM8728^T; lane C, the spent medium of AH4. The arrows on the left and middle TLC indicate spots of lactose released from the degraded SL molecules, while the arrow on the right TLC indicates a spot of lactose initially added to the medium. The upper spots on all A lanes and the uppermost spots on lanes A, B, and C were some unknown ingredients of the broth medium we used, as they disappeared when we conducted TLC on the milk oligosaccharides dissolved in PBS (data not shown).

Morphological and biochemical characteristics of the 3′-SL-degrading isolates

Based on the results of Gram staining, the isolates were gram-positive cocci of approximately 1 μm in diameter, which was comparable to the findings for *E. gallinarum* JCM8728^T (data not shown). Using the Rapid ID 32 Strep System, the isolates were identified as *E. gallinarum,* as they exhibited almost identical biochemical characteristics as the type strain of the species (Table 1). However, our additional TLC tests revealed that the type strain could not degrade either 3′-SL or 6′-SL, whereas the isolates degraded both sugars, as indicated by the presence of additional lactose spots (Fig. 3a and b). Interestingly, both the type strain and isolates utilized lactose almost completely, as indicated by the disappearance of original lactose spots (Fig. 3c), which is consistent with the results presented in Table 1, which show that lactose was degraded by all *E. gallinarum* strains tested.

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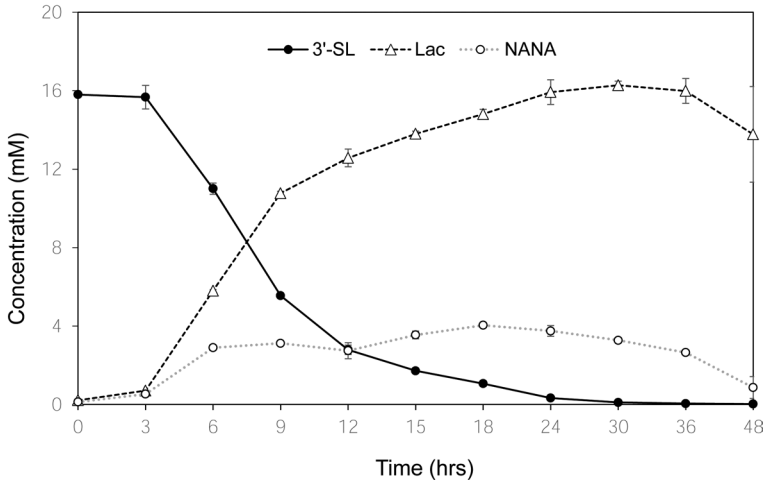

Fig. 4. Quantitative high-performance liquid chromatographic analysis presenting the time course of the concentrations of lactose (Lac) and *N*-acetylneuraminic acid (NANA) generated during 3′-sialyllactose (3′-SL) degradation by strain AH4. Experiments were performed in triplicate. The results are presented as arithmetic means, and error bars denote standard deviations.

Mode of 3′-SL degradation by strain AH4

Our HPAEC-PAD analysis of culture supernatants collected at different points during the time course demonstrated that strain AH4 completely degraded 16 mM (approximately 1%) 3′-SL after 30 hr and released lactose and NANA. The amount of liberated lactose matched the amount of degraded 3′-SL, whereas that of NANA was approximately one-fourth of the expected amount. The concentrations of both metabolites declined gradually after 30 hr of incubation (Fig. 4).

DISCUSSION

In the present study, we successfully isolated 3′-SL-degrading bacteria from the intestinal contents of suckling rats using an enrichment technique essentially as described by Jackson *et al.* [22]. The technique was designed to isolate 3′-SL-degrading bacteria, if any, with smaller numbers of concomitant non-3′-SL-degrading bacteria at the start of enrichment. In this way, the former could have an extra nutritional advantage over the latter during enrichment, permitting them to form well-separated colonies on a streaked agar plate for easy selection.

Our genomic analysis assigned all 3′-SL-degrading isolates taxonomically to *E. gallinarum*, which is a common inhabitant in the intestines of both humans and animals [23]. The additional phenotypic analysis revealed that, unlike the type strain of the species, the isolates could degrade both 3′-SL and 6′-SL. Both oligosaccharides are degraded by enzymes collectively termed sialidases, which are widely distributed in higher animals and various microorganisms inclusive of bacteria [24]. Concerning bifidobacteria, the enzymes produced by *B. longum* subspecies *infantis* [25] and *B. bifidum* [26] have been well studied because these species are particularly abundant among the gastrointestinal microbes of breastfed human infants [10], thereby promoting the health of the host [27]. Such a symbiotic relationship has never been described in any other mammal to our knowledge, and thus, this is the first report to suggest a rodent version of symbiosis in which the enterococci that produce sialidase act in a similar manner as bifidobacteria in humans. In this connection, we undertook an *in-silico* search for any putative sialidase gene of *E. gallinarum* reported in the NCBI database (https://www.ncbi.nlm.nih.gov/) and found that a human clinical isolate of *E. gallinarum* FDAARGOS, the FDAARGOS 163 strain, had a gene encoding putative exo-alpha-sialidase (QGR83607.1). Further study is in progress to determine whether such a gene is present in our *E. gallinarum* isolates and whether the gene encodes the enzyme in question.

In the present study, we did not investigate whether the infant rats, like human infants, develop a microbiota dominated by the milk oligosaccharide–degrading enterococci during suckling. However, this seems unlikely to take place because a recent metagenomics study [28] showed that lactobacilli, not enterococci, were major members of the gut microbiota of suckling rats. We thus conducted a time-course study to provide a possible reason for the marked increase of lactobacilli and found that *E. gallinarum* AH4 degraded 3′-SL to release NANA and lactose during growth. Interestingly, the strain partially utilized the released NANA, whereas the released lactose molecules were left intact, although it did utilize lactose when cultured with lactose as the sole source of carbohydrates. Regarding this finding, Nishiyama *et al.* [29] demonstrated in their co-culture experiment that a sialidase-positive *B. bifidum* strain degraded 6′-SL to “cross-feed” a sialidase-negative *B. breve* strain. Correspondingly, Gotoh *et al.* [30] demonstrated that *B. bifidum* degraded HMOs to cross-feed other members of bifidobacteria (i.e., *B. longum* and *B. breve*) and develop bifidobacteria-rich communities in the infant gastrointestinal tract. More recently, Centanni *et al.* [31] reported that one of the *B. bifidum* strains hydrolyzed 2’-*O*-fucosyl-lactose, a major fucosylated human milk oligosaccharide, but did not use fucose released into the culture medium, which was in turn utilized by a concomitant *B. breve* strain. Moreover, Pastell *et al.* [32] demonstrated that *B. adolescentis* and *B. longum* strains were able to degrade arabinoxylo-oligosaccharides but that the former and the latter preferentially consumed the xylo-oligosaccharides and arabinose released, respectively. These findings suggest that, while highly speculative, the enterococci degrade both 3′-SL and 6′-SL to consume preferentially NANA while cross-feeding “altruistically” the released lactose to other concomitant members

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of the infant rat microbiome, lactobacilli for example, serving a similar function as the bifidobacterial community in the human gastrointestinal tract.

Meanwhile, it has been demonstrated in laboratory animals that NANA and its oligosaccharide conjugates are essential nutrients for brain development. For example, Tarr *et al.* [33] reported that mice fed 3′-SL or 6′-SL exhibit an alteration of their gastrointestinal microbiome and acquire tolerance to social disruption, presenting evidence supporting the notion of the gastrointestinal-brain axis via dietary SL. More recently, Oliveros *et al.* [34] reported that adult rats fed 6′-SL displayed better cognitive ability than control animals. The present study thus suggests that bacteria with sialidase activity, such as *E. gallinarum* in rats and yet to be identified bacteria in mice, an interesting topic for future research, play critical roles in the beneficial effects of 3′-SL or 6′-SL on the host. As NANA and its oligosaccharide conjugates have been increasingly recognized as being similarly beneficial in humans [35, 36], bifidobacteria with sialidase activities in the gastrointestinal tract may play roles of comparable importance. This, in turn, points to the novel prospect that clarifying the mechanism by which these sialidase-positive gastrointestinal bacteria benefit health and welfare in laboratory rodents will be useful for further substantiating the importance of the symbiosis between humans and bifidobacteria.

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