

# Persistence of Orally Administered *Lactobacillus* Strains in the Gut of Infant Mice

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The present study tested the persistence of orally administered bacteria in the gut of suckling mice. We used three bacterial strains: one strain of *Lactobacillus johnsonii* (designated strain Ms1) that was previously isolated from the mouse stomach, and two strains of *L. plantarum*, (strain No. 14 and JCM 1149<sup>T</sup>). We detected *L. johnsonii* Ms1, but neither strain of *L. plantarum*, in the gut 7 days after administration when the organisms were administered on days 0, 1, 3 or 7 of neonatal life. None of the strains was detected in the gut 7 days after the administration on days 14 or 28 of neonatal life. *L. johnsonii* Ms1 and *L. plantarum* JCM 1149<sup>T</sup> exhibited similar levels of *in vitro* association with gut tissues, with both strains showing association that was significantly higher than that of *L. plantarum* No. 14. In a separate experiment, the number of total bacteria and lactobacilli in the gut, as estimated by real-time quantitative PCR, was significantly higher in 14- and 21-day-old mice than in 0- and 7-day-old mice. In addition, the number of total bacteria was higher in 21-day-old mice than in 14-day-old mice, and the number of lactobacilli was higher in 7-day-old mice than in 0-day-old mice. These results suggest that gut persistence of administered bacteria in infant mice is species- or strain-specific and is affected by the development of indigenous microbiota. In addition, gut persistence of administered bacteria may not always depend on the tissue association capacity.

**Key words:** Probiotics; *Lactobacillus plantarum*; *Lactobacillus johnsonii*; colonization; mice

## INTRODUCTION

Human and animal studies have revealed that exogenously administered bacteria can pass into the feces without colonization of the host intestine [1-8]. Colonization resistance for administered bacteria is attributable to several environmental stresses, including gastric acid, pancreatic enzymes, bile, indigenous bacteria, mucus layers, and intestinal immunity. Considering that the gut in infancy is relatively benign in terms of these stresses, the ability of probiotics to colonize the gut may be higher in infancy. Previous studies showed that the administration of lactobacilli and bifidobacteria to pregnant women and animals results in the colonization of administered bacteria in the gut of vaginally delivered offspring [9–13]. These findings suggest mother-to-infant transfer of administered bacteria, while confirming the higher ability of administered bacteria to colonize the infant gut.

We previously reported that daily intragastric administration of *Lactobacillus plantarum* strain No. 14

reduces adipocyte size in diet-induced obese mice [14]. In addition, Nagata et al. reported a small-scale, randomized, placebo-controlled, double-blind trial that showed a body-fat-reducing effect of *L. plantarum* No. 14 in healthy female volunteers [15]. These findings suggest that this bacterial strain represents a potential candidate probiotic for the prevention of obesity. In addition, we observed that the fecal recovery of viable *L. plantarum* No. 14 peaked at 6 h and decreased to undetectable levels at 48 h after administration in fasted adult mice [16, 17]. In contrast, *Lactobacillus johnsonii*, which we previously isolated from the mouse stomach (and here designate as strain Ms1) remained detectable for at least 120 h after administration. Therefore, *L. plantarum* No. 14, a strain nonindigenous to the mouse intestine, has reduced persistence in the gut of adult mice compared with indigenous *L. johnsonii* Ms1. The present study tested whether nonindigenous bacterial strains persist in the gut when the organisms are administered in infancy.

## MATERIALS AND METHODS

### *Animal handling and experimental design*

Eight-week-old male and 7-week-old female ICR

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mice were purchased from Japan SLC (Hamamatsu, Japan) and housed in plastic cages in a temperature-controlled ( $23 \pm 2^\circ\text{C}$ ) room with a dark period from 20:00 to 08:00. Mice were allowed free access to tap water and a synthetic diet prepared according to AIN-93G guidelines [18]. In individual cages, individual males were mated with up to three females; the resulting pregnant mice were housed individually. Day of birth was referred to as day 0 of neonatal life. Offspring and dams were housed in the same cage until sacrifice. On days 0, 1, 3, 7, 14 or 28 of neonatal life, seven offspring in each litter were administered orally  $10 \mu\text{l}$  of phosphate-buffered saline (PBS) containing  $10^6$  colony forming units (CFU) per mouse of either *L. plantarum* No. 14, *L. plantarum* JCM 1149<sup>T</sup> or rifampicin-resistant *L. johnsonii* Ms1. Inocula were prepared as described below. In total, 126 offspring from 18 dams were used for this experiment. Oral administration was performed using a  $10\text{-}\mu\text{l}$  low retention pipette tip (Molecular BioProducts, San Diego, CA, USA) connected to an autopipette (Eppendorf, Tokyo, Japan). From each litter, 1 and 6 offspring on 1 day and 7 days after administration, respectively, were anesthetized by diethyl ether and euthanized by decapitation. After a laparotomy, the entire intestinal tract and contents were excised, and samples were subjected to enumeration of administered bacteria.

In a separate experiment, offspring without *Lactobacillus* administration were similarly anesthetized and killed on days 0, 7, 14 or 21 of neonatal life. After a laparotomy, the entire intestinal tract and contents were excised, and samples were snap-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  for RNA extraction.

For the *in vitro* association experiment, 12-week-old female ICR mice were purchased from Japan SLC and maintained as described above. After acclimatizing to the synthetic diet for 1 week, mice were allowed free overnight access to tap water supplemented with  $1 \text{ mg/ml}$  ampicillin sodium. Mice were then anesthetized and euthanized as above. After a laparotomy, the stomach, small intestine, cecum, and colon were excised, and the luminal contents were washed out with ice-cold PBS. A tissue disc (approximately  $3 \text{ mm}$  in diameter) was excised from each tissue sample and subjected to the *in vitro* association experiment.

This study was approved by the Hokkaido University Animal Use Committee (approval no. 08-0139), and the animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

#### *Maintenance and enumeration of Lactobacillus strains*

*L. plantarum* No. 14, a strain previously isolated from pickled shallots [15], was donated by the Research Center of Momoya Co., Ltd. (Saitama, Japan). *L. plantarum* JCM 1149<sup>T</sup>, originally isolated from pickled cabbage, was obtained from the RIKEN BioResource Center (Tsukuba, Japan). *L. johnsonii* Ms1 was previously isolated from the mouse stomach in our laboratory [16]. These bacteria were cultured in de Man Rogosa Sharpe (MRS) broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at  $37^\circ\text{C}$  for 14 hr. The bacteria were harvested by centrifugation at  $3,000 \times g$  for 20 min, washed once with PBS, and then resuspended in PBS for oral administration and the *in vitro* association experiment. In order to specifically detect the administered *L. johnsonii* Ms1 in mouse gut tissue, rifampicin-resistant *L. johnsonii* Ms1 was prepared according to Frece et al. [19].

Tissue samples were homogenized in  $450 \mu\text{l}$  of anaerobic phosphate buffer. A dilution series was prepared, and  $30 \mu\text{l}$  of each dilution was plated onto solid medium. The number of *L. plantarum* cells was measured using *L. plantarum*-selective medium [20]. The number of rifampicin-resistant *L. johnsonii* Ms1 cells was measured using MRS agar supplemented with  $100 \mu\text{g/ml}$  rifampicin. The plates were incubated at  $37^\circ\text{C}$  for 2 days under anaerobic conditions using the AnaeroPack system (Mitsubishi Gas, Tokyo, Japan), and single colonies were counted. The total number of bacteria per gram of tissue was calculated; values are presented as logarithmic CFU.

#### *In vitro association experiment*

After washing three times with PBS, tissue disc samples were added to  $1 \text{ ml}$  PBS containing  $10^8$  CFU of either *L. plantarum* JCM 1149<sup>T</sup>, *L. plantarum* No. 14, or rifampicin-resistant *L. johnsonii* Ms1 and incubated at  $37^\circ\text{C}$  for 30 min with gentle rotation. Tissue samples were then washed three times with PBS and homogenized in  $1 \text{ ml}$  of PBS. The homogenate was subjected to enumeration of viable bacteria by using selective media as described above.

Additionally, *L. plantarum* No. 14 and *L. johnsonii* Ms1 were stained with PKH26 (Sigma, St. Louis, MO, USA) and 5-(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, Enzo Life Science, Farmingdale, NY, USA), respectively, as previously described [16]. Using a mixture of these bacteria, an *in vitro* association experiment was performed as described above. The resulting tissue homogenate was applied to a glass slide, dried at room temperature, mounted using

ProLong Gold Antifade Reagent with DAPI (Molecular Probes, Tokyo, Japan), and observed under a fluorescence microscope.

#### Enumeration of total bacteria and lactobacilli in the gut

Total RNA was extracted from tissue samples using TRizol reagent (Invitrogen, Tokyo, Japan) as previously described [21]. After removing genomic DNA by treating with DNase, approximately 15 ng of total RNA was annealed with 100 pmol of primer L1401 [22] at 65°C for 5 min, and first strand cDNA was synthesized using a SuperScript First-Strand Synthesis System (Invitrogen) according to the instructions of the manufacturer. Samples were then treated using a QIAquick PCR Purification Kit (Qiagen, Tokyo, Japan) according to the instructions of the manufacturer.

The numbers of total gut bacteria and lactobacilli were estimated by real-time quantitative PCR (RT-qPCR). Specifically, the first strand cDNA samples (10 ng) were added to a 25- $\mu$ l reaction mixture containing 12.5  $\mu$ l SYBR Premix Ex Taq (Takara) and 200 nM each of primers U968 and L1401 (to assay for total bacteria [22]) and LactoF and LactoR (to assay for lactobacilli [23]). The reaction conditions were 95°C for 10 sec, followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec. The fluorescent products were detected at the last step of each cycle. A melting curve analysis was performed after amplification to distinguish the targeted PCR product from the nontargeted PCR product. All samples were analyzed in duplicate. *Lactobacillus murinus* (JCM 1717<sup>T</sup>) was used for preparing the standard for RT-qPCR as previously described [24].

#### Statistics

Results are presented as means  $\pm$  SEM. Comparisons were performed using one-way ANOVA followed by the Tukey-Kramer test. GraphPad Prism for Macintosh (version 5.0, GraphPad Software, Inc., San Diego, CA, USA) was used for the analyses.

### RESULTS

In order to test whether orally administered bacteria persist in the gut of suckling mice, gut tissue at one and seven days after bacterial administration was subjected to culture using selective media. We confirmed the absence of detectable levels of viable rifampicin-resistant *L. johnsonii* Ms1 and *L. plantarum* in the feces of pregnant and lactating mice (data not shown). In addition, our preliminary experiment showed that viable rifampicin-resistant *L. johnsonii* and *L. plantarum* were not detected in suckling mice that had not been

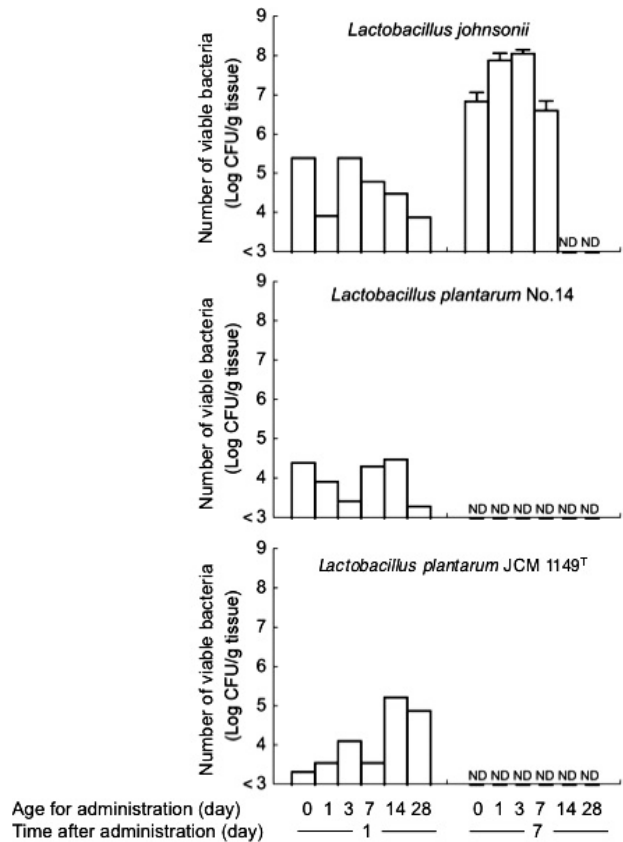


Fig. 1. Recovery of orally administered bacteria from the gut of suckling mice.

*L. johnsonii*, *L. plantarum* No. 14 and *L. plantarum* JCM 1149<sup>T</sup> (upper, middle, and lower panels, respectively) were administered on day 0, 1, 3, 7, 14 or 28 of neonatal life, and the number of viable bacteria in the entire intestinal tract was determined 1 day (1 mouse per group) and 7 days (6 mice per group) after administration. Values are presented as means  $\pm$  SEM. ND, not detected.

administered bacteria (data not shown). One day after administration, we successfully detected the administered bacteria in the gut tissue of mice to which the bacteria were administered on day 0, 1, 3, 7, 14 or 28 of neonatal life (Fig. 1). Seven days after administration, rifampicin-resistant *L. johnsonii* Ms1 was detected in the gut tissue of all mice when the organisms were administered on day 0, 1, 3, or 7 of neonatal life. When the organisms were administered on day 14 or 28 of neonatal life, however, rifampicin-resistant *L. johnsonii* Ms1 was no longer detected in the gut 7 days after administration. In contrast, no detectable levels of *L. plantarum* No. 14 or *L. plantarum* JCM 1149<sup>T</sup> were found in the gut tissue of mice 7 days after administration, regardless of the age at administration.

In order to test whether the distinct persistence of *L.*

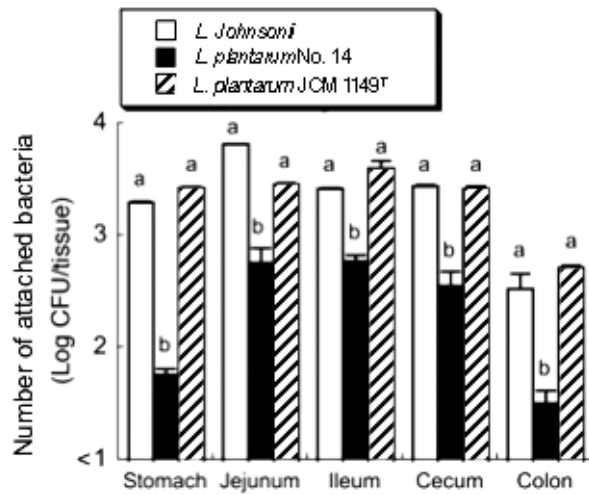


Fig. 2. Recovery of viable bacteria following *in vitro* association with gut tissues.

*L. johnsonii*, *L. plantarum* No. 14 and *L. plantarum* JCM 1149<sup>T</sup> were added to tissue samples excised from the stomach, jejunum, ileum, cecum, and colon of adult mice that had been administered antibiotics in drinking water overnight. After incubation at 37°C for 30 min, tissue samples were washed with PBS and then subjected to enumeration by culture. Values are presented as means  $\pm$  SEM of six mice per group. For each tissue, values not sharing the same letters are significantly different at  $p < 0.05$ .

*johnsonii* Ms1 and *L. plantarum* in the gut of suckling mice is related to the tissue association capacity of the bacteria, we performed *in vitro* association experiments. In the gut tissue samples excised from the stomach, jejunum, ileum, cecum and colon of mice administered overnight antibiotics in drinking water, no detectable levels of culturable anaerobes were found (data not shown). Samples of these tissues were then incubated with *Lactobacillus* bacteria for 30 min. The number of tissue-associated bacteria per tissue disc did not differ significantly between *L. johnsonii* Ms1 and *L. plantarum* JCM 1149<sup>T</sup> (Fig. 2). However, the number of *L. plantarum* strain No. 14 bacteria associated with each of the tissues was significantly lower than those of *L. johnsonii* Ms1 and *L. plantarum* JCM 1149<sup>T</sup> in the respective tissue. Following incubation with bacteria, the tissue homogenates were subjected to staining and microscopic evaluation, which revealed a number of CFSE-stained *L. johnsonii* Ms1 (Fig. 3). In equivalent experiments, PKH26-stained *L. plantarum* No. 14 was rarely observed. These results suggest that the lower number of tissue-associated *L. plantarum* No. 14 bacteria detected by culturing (Fig. 2) is not due to reduced viability of *L. plantarum* No. 14 during incubation with gut tissues.

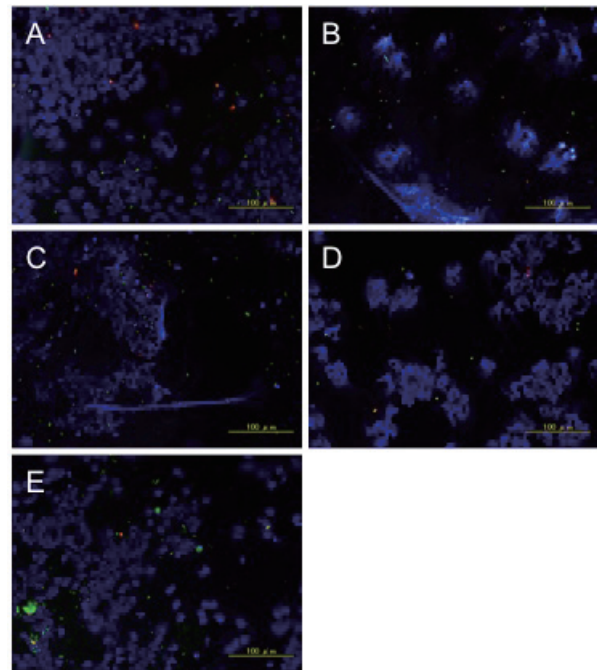


Fig. 3. Microscopic observation of bacteria associated with the gut tissues *in vitro*.

A mixture of CFSE-stained *L. johnsonii* (green) and PKH26-stained *L. plantarum* No. 14 (red) was added to tissue samples excised from the stomach, jejunum, ileum, cecum, and colon (charts A, B, C, D and E, respectively) of adult mice administered antibiotics in drinking water overnight. After incubation at 37°C for 30 min, tissue samples were washed with PBS, homogenized, stained with DAPI (blue) and then observed using fluorescence microscopy.

The development of indigenous gut microbiota may affect gut colonization by administered bacteria. Therefore, we estimated the numbers of total bacteria and lactobacilli in the gut of 0-, 7-, 14- and 21-day-old mice by RT-qPCR for 16S rRNA genes. With this assay, there were no significant differences in the number of total bacteria between 0- and 7-day-old mice, while the number subsequently increased with age (Fig. 4). The number of total bacteria was significantly higher in 14-day-old mice than in 0- and 7-day-old mice, and the number in 21-day-old mice was significantly higher than that in 14-day-old mice. The number of lactobacilli was significantly higher in 14- and 21-day-old mice than in 0- and 7-day-old mice, and the number in 7-day-old mice was significantly higher than that in 0-day-old mice.

## DISCUSSION

The present study hypothesized that nonindigenous bacterial strains may colonize the gut when the organisms are administered in infancy. To test this idea,

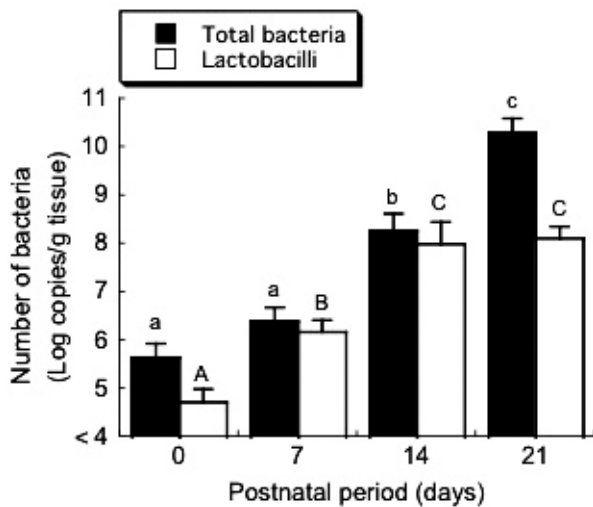


Fig. 4. Time-course changes in the numbers of total bacteria and lactobacilli in the gut tissue of suckling mice.

Total RNA was isolated from the tissues of the entire intestinal tract excised from 0-, 7-, 14- and 21-day-old mice, and the number of bacteria was estimated by RT-qPCR. Values are presented as means  $\pm$  SEM of six mice per group. Total numbers of bacteria and lactobacilli not sharing the same letters are significantly different at  $p < 0.05$ .

two strains of *L. plantarum* were administered directly to infant mice. Our results indicate that both strains of *L. plantarum*, when administered on day 0 of neonatal life, failed to persist in the gut. *L. plantarum* is found in fermented food products derived from plant material and is also a natural inhabitant of the human gut [4, 25–29]. However, previous studies reported no detectable levels of *L. plantarum* naturally occurring in the gut of piglets [30] or mice [16, 17]. We therefore suspected that *L. plantarum* might be adapted to the gastrointestinal tract of humans but not to those of other animals. Indeed, gut colonization by some bacterial species depends on host species. For instance, *Helicobacter pylori*, an indigenous species in the human gut, is maladapted to the gut of experimental animals [31].

The intestinal tract is sterile *in utero*, and the colonization process begins as the neonate is exposed to bacteria in the environment, birth canal, maternal feces and other sources during and after birth [13, 32–34]. Intestinal microbiota thus develop rapidly after birth and are initially strongly dependent on maternal fecal and vaginal microbiota. In the present study, the numbers of total bacteria and lactobacilli, as estimated by RT-qPCR, in the gut of suckling mice increased with neonatal age following 7 days post birth, whereas the number of total bacteria was not significantly different between 0-day-

old mice and 7-day-old mice. It thus appears likely that the gut was already populated by indigenous gut microbiota on day 0 of neonatal life, the first time point at which we administered bacteria to the infant mice. Considering that probiotics administered to pregnant women and animals colonized the gut of vaginally delivered neonates [9–13], we presume that *L. plantarum* could have colonized the gut if the bacteria had been administered to the pregnant mice or to the neonates immediately after birth. In our preliminary experiment, in which abundant numbers of *L. plantarum* No. 14 bacteria ( $10^9$  CFU) were intragastrically administered every day to pregnant and lactating mice, we detected viable *L. plantarum* No. 14 in the feces of dams in numbers ( $10^8$  CFU/g feces) that were comparable to those seen after inoculation with *L. johnsonii*. Under these conditions, however, we failed to detect viable organisms in the gut of the offspring (unpublished data). In another preliminary experiment, pregnant and lactating mice were administered with antibiotics (i.e., a mixture of ampicillin sodium, neomycin sulfate, and vancomycin hydrochloride) in drinking water. The levels of total aerobes and anaerobes were below our assays' limit of detection in the feces ( $10^3$  CFU/g feces) of these mice, suggesting reduced bacterial exposure of the offspring. Under these conditions, *L. plantarum* No. 14 administered daily to the pregnant and lactating mice was still undetectable in the gut of offspring (unpublished data). These results suggest that *L. plantarum* No. 14 fails to persist in the infant gut even when the organisms are administered maternally to pregnant and lactating mice. It therefore appears unlikely that resistance by rapidly developing indigenous bacteria after birth is responsible for unsuccessful persistence of *L. plantarum* No. 14 and JCM 1149<sup>T</sup> in the infant gut.

In the present study, we detected rifampicin-resistant *L. johnsonii* Ms1 in the gut of suckling mice at 7 days after bacterial administration when the organisms were administered on day 0, 1, 3 or 7 of neonatal life. Because this strain was previously isolated from the mouse stomach in our laboratory [16], the persistence of this strain in the gut of suckling mice is not surprising. However, our study tracked this strain in the gut only up to 7 days after administration; it remains unclear whether this strain colonized the gut in the longer term. Notably, this strain was not detected in the gut 7 days after administration when the organisms were administered in 14- or 28-day-old mice. We presume that the development of the gut immune system in the host, along with the increased population of indigenous gut bacteria in late infancy, might preclude the persistence of

administered bacteria. In our previous study, in which 5-week-old mice were administered rifampicin-resistant *L. johnsonii* Ms1 after overnight food deprivation, the bacterium remained detectable for at least 5 days after administration [16]. Therefore, dietary inputs might affect the gut persistence of administered bacteria in 14- and 28-day-old mice in the present study.

The present study examined whether the different persistence of *L. johnsonii* Ms1 and *L. plantarum* in the gut of suckling mice is associated with the tissue association capacity of the bacteria. *In vitro* association studies showed that the numbers of bacteria associated with the gut tissues were similar for *L. johnsonii* Ms1 and *L. plantarum* JCM 1149<sup>T</sup>. Because *L. plantarum* JCM 1149<sup>T</sup>, as well as strain No. 14, failed to persist in the gut of suckling mice, the ability of bacteria to persist in the gut may not correlate with tissue association capacity. However, the present study used the adult gut tissues for the *in vitro* association experiments, while the *in vivo* persistence experiments were performed in infant mice, and the tissue association capacity of bacteria might differ between infant and adult mice. In addition, although the *in vitro* association of *L. plantarum* No. 14 with gut tissues was significantly lower than that of *L. johnsonii* Ms1 and *L. plantarum* JCM 1149<sup>T</sup>, the basis of this difference remains unclear. Further studies are needed to clarify the relationship between the tissue colonization and tissue association of bacteria in the gut.

In conclusion, the present findings suggest that gut persistence of administered bacteria in infant mice is specific to the bacterial species and/or strain. Although indigenous bacteria appear more persistent in the infant gut, this persistence presumably reflects the development of indigenous microbiota, host immunity, and dietary inputs. Furthermore, the gut persistence of administered bacteria may not always depend on the tissue association capacity.

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